The effect of mutated cysteine residues in von Willebrand factor

Anna Pernilla Tjernberg

The front and back cover show an artistic impression of von Willebrand factor (VWF) multimers seen through a kaleidoscope. The impression was generated from multimer patterns of normal pooled plasma VWF, recombinant wild-type VWF, and wild-type VWF co-expressed with C2773S VWF as shown in Figure 1B of Chapter 5. Design by Sjoerd van den Worm.

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"Prime numbers are what is left when you have taken all the patterns away. I think prime numbers are like life. They are very logical but you could never work out the rules, even if you spent all your time thinking about them."

Christopher John Francis Boone in
"The curious incident of the dog in the night-time"
by Mark Haddon

Till mina nära och kära!

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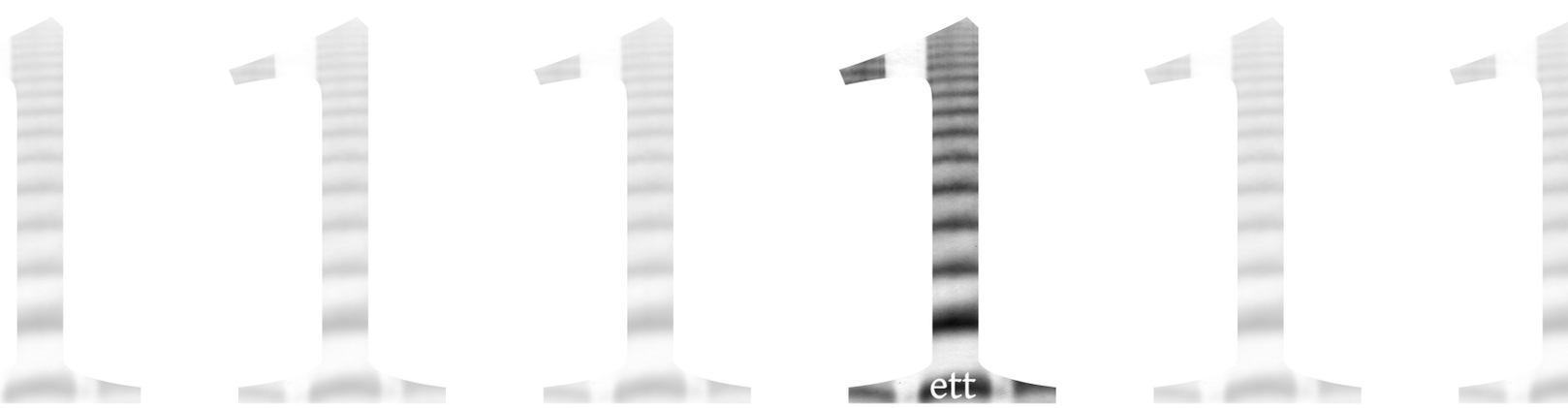

Introduction

The delicate balance of coagulation and fibrinolysis allows blood to flow free through the vasculature to transport oxygen and nutrients, and to remove waste products. If the intricate systems that regulate primary hemostasis, and the procoagulant, anti-coagulant and fibrinolytic activities are not finely balanced there is a risk for thrombotic or hemorrhagic events. Our genetic makeup as well as environmental factors can influence this balance. In primary hemostasis, von Willebrand factor (VWF) plays a crucial role in the formation of the platelet plug during the initial response of sealing off a wound to the vasculature. It performs this task by functioning as a molecular glue between platelets and exposed subendothelial structures of the vasculature. A shift in the level of VWF or an alteration in its structure may result in a bleeding disorder called von Willebrand disease (VWD). In this thesis the effect of mutations of cysteine residues in VWF on the synthesis of VWF was studied in a model system and compared with the phenotype observed in patients with VWD that carry these mutations.

Discovery of a new disease and a new factor

Eighty years ago, the first report on "hereditary pseudohemophilia" was published by the Finnish MD Erik von Willebrand (1,2). He studied three families with a total of 66 members from Åland. The distribution between sexes (16 females and 7 males), the slightly decreased to normal number of platelets, the prolonged bleeding time, and the severe mucocutaneous bleeding, which contrasted with the spontaneous deep tissue bleeding or bleeding of the joints observed in true hemophilia led von Willebrand to separate this disorder from hemophilia. He called it "hereditary pseudohemophilia". In honour of its discoverer, the disease was given his name and today is known as von Willebrand disease.

The protein mutated in VWD that distinguishes it from true hemophilia remained elusive and the observation of low factor VIII (FVIII) in patients with VWD made matters even more confusing. However, the revolutionary discovery that FVIII forms a dissociable complex with the protein now known as VWF, provided a possible explanation for the occurrence of the two different bleeding disorders, VWD and hemophilia (3). Research on VWD progressed rapidly after the

purification of VWF (4), and the cloning and sequencing of the cDNA and the complete VWF gene (5-10). Together with direct sequence analysis of part of the VWF protein (11), this led to the prediction of the amino acid sequence of the VWF protein. These milestones accelerated the progress of unraveling the diverse properties of this complex protein and its role in hemostasis.

Biosynthesis of VWF

Over the years, knowledge has accumulated on the biosynthesis and the structure and function of VWF. We now know that synthesis of VWF is restricted to endothelial cells (12,13) and megakaryocytes (14). The study of cultured endothelial cells revealed two distinct pathways of secretion for VWF, constitutive and regulated secretion (reviewed by Wagner (15) and Sadler (16)).

Biosynthesis of VWF is a complex process that requires extensive post-translational modification of the newly synthesized precursor to yield high molecular weight (HMW) multimers ranging from 500 to 20.000 kDa (15,16). Synthesis of HMW multimers starts with transcription of the ~178 kbp VWF gene (10), which is located on the short arm of chromosome 12 (17,18). The 52 exons of the VWF gene (Fig. 1) are transcribed into a ~9 kb mRNA (10). Translation of the VWF mRNA starts at exon 2 and yields a 2813 amino acid (aa) long single chain precursor protein, preproVWF, with an approximate molecular weight of 350.000 (10). Exon 2 of *VWF* codes for the signal peptide (22 aa), whereas exons 3-17 encode the large VWF-propeptide (741 aa) (10), which is also known as von Willebrand antigen II (19). The remaining exons, 18-52, encode the mature VWF subunit (2050 aa) (10).

Examination of the amino acid sequence of preproVWF revealed that VWF consists of several repeated domains (A-D, Fig. 2) (8,9). Over 90% of the amino acid residues of preproVWF have been mapped to such domains (8). In addition, the amino acid sequence appeared to contain an unusual high number of cysteine residues (8.2%) (20). It was shown that all these cysteine residues contributed to the formation of either intrachain or interchain disulfide bonds (20). Cysteines are mainly found at the N- and C-terminal regions of mature VWF (11). The former was shown to be necessary for multimerization of VWF; the latter for dimerization (Fig. 2) (21,22).

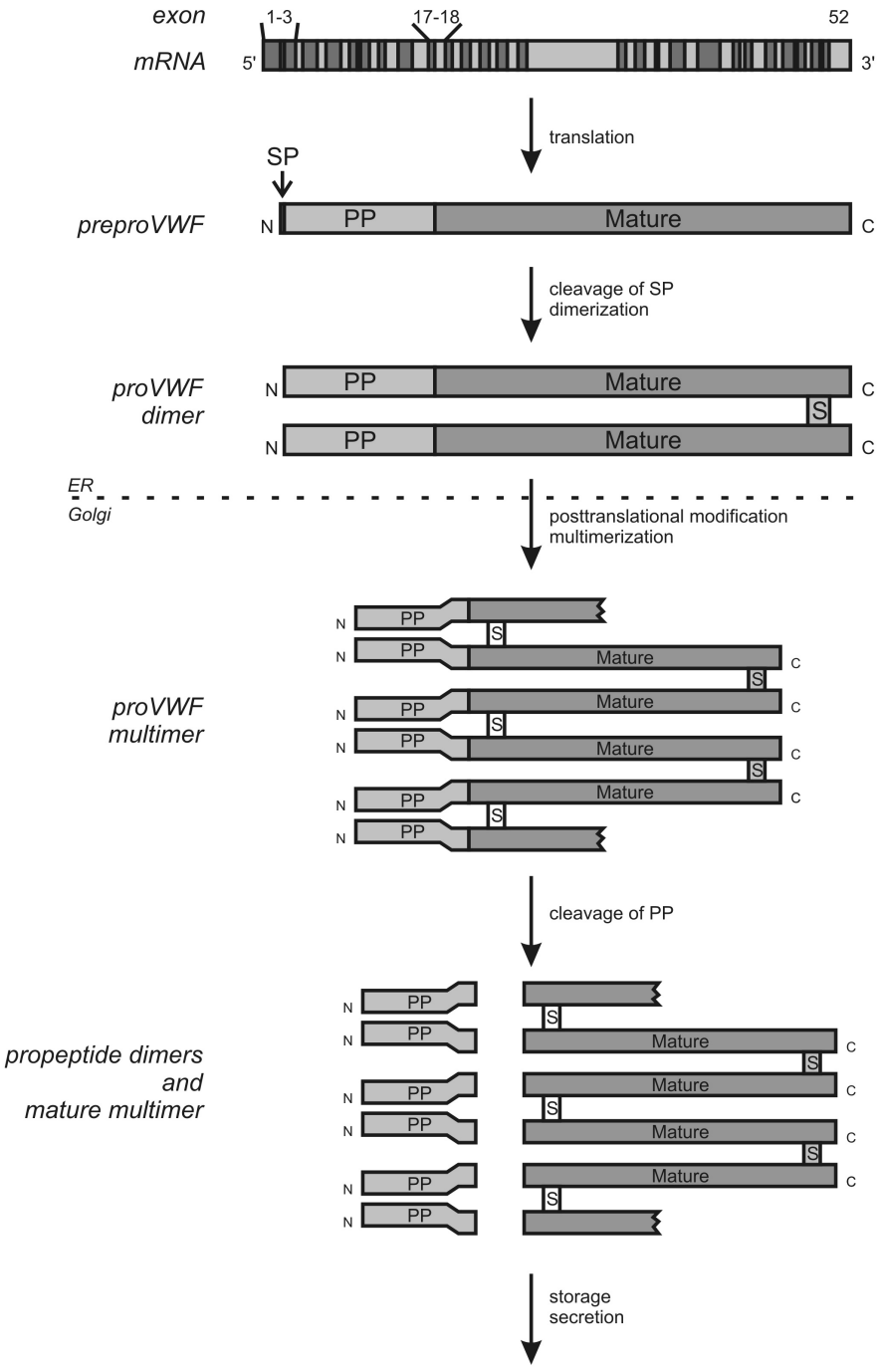

Fig. 1, schematic overview of the exon distribution in VWF mRNA, its translation to preproVWF and the subsequent processing steps. Important steps are removal of the signal peptide (SP), dimerization and multimerization of proVWF, and cleavage of the VWF-propeptide (PP) resulting in mature multimers lacking the PP. Cysteine disulfide bonds formed either at the C-terminus (dimerization) or the N-terminus (multimerization) are indicated with an "S". See text for further details.

Dimerization and multimerization of VWF

During the assembly of monomeric VWF to high molecular weight multimers, its routing through two intracellular compartments, the endoplasmic reticulum (ER) and the Golgi apparatus, are crucial for dimerization and multimerization, respectively (Fig. 1). The signal peptide targets preproVWF to the ER. After translocation of preproVWF into the ER the signal peptide has served its purpose and is cleaved off between aa 22 and 23 (Fig. 2). In the ER, selected asparagine (Asn) residues of proVWF are glycosylated by addition of high mannose oligosaccharide chains, a process that is a prerequisite for the subsequent formation of disulfide linked proVWF (23). The proVWF dimers are formed through interchain disulfide bond(s) between cysteine residues located in the 90 carboxy-terminal residues of VWF, also known as the cystine knot (CK) domain (20,24). The dimerization of proVWF generates the first building block in the formation of HMW multimers of VWF. These building blocks are transported to the Golgi apparatus, where the Asn-linked oligosaccharides are processed and subsequently sulfated (25); where *O*-linked oligosaccharides are added to serine and threonine residues; and where the formation of HMW multimers occur (15).

Multimerization of VWF involves the formation of interchain disulfide bonds between cysteine residues in the D3 domains of two proVWF dimers. This domain is located at the N-terminus of the proVWF dimer (Fig. 1). Continuous addition of building blocks results in a growing proVWF multimer. After removal of the VWF-propeptide, comprising the D1 and D2 domains, by cleavage after residue 763 (Fig. 2), the multimer consists of an even number of mature VWF subunits (Fig. 1). The removal of the VWF-propeptide from proVWF has been suggested to occur simultaneously with the assembly of proVWF dimers to multimers (26) and is most likely catalysed by furin, a subtilisin-like serine protease (27-29). Multimerization of VWF in the Golgi apparatus is dependent on the presence of the VWF-propeptide (30,31). However, removal of the VWF-propeptide is not required for the formation of multimers (32).

On the basis of these results and the identification of the active site sequence of disulfide isomerase, CXXC, in each of the D domains of the VWF-propeptide (33), the VWF-propeptide was suggested to have a dual role in the multimerization of VWF. Firstly, it was proposed to recognize and align N-terminal regions of VWF dimers (30,31) and, secondly, it was proposed to

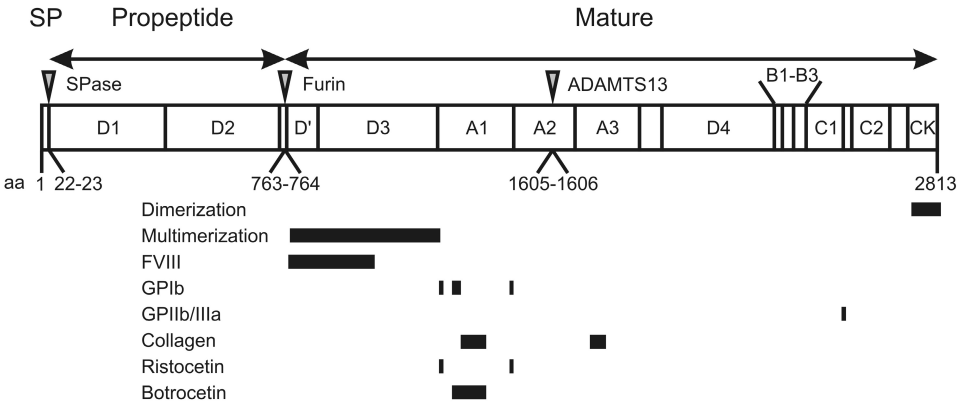

Fig. 2, overview of preproVWF and the repeated A-D regions. A signalpeptidase (SPase) removes the signal peptide (SP, aa 1-22) after translocation of preproVWF into the ER. In the Golgi apparatus, furin releases the VWF-propeptide (aa 23-763) from proVWF multimers resulting in VWF multimers (aa 764-2813). ADAMTS13 cleaves VWF multimers between aa 1605 and 1606. Indicated are domains involved in the formation of HMW multimers (dimerization, CK domain aa 2724-2813; and multimerization, D'-D3 domains aa 769-1242), domains involved in primary (GPIb, GPIIb/IIIa and collagen) and secondary hemostasis (FVIII), as well as functional domains that are important for *in vitro* testing of VWF (binding of ristocetin and botrocetin). Binding sites (aa): FVIII 764-1035; GPIb 1237-1251, 1277-1305, 1458-1471; GPIIb/IIIa 2507-2509; collagen 1305-1385, 1711-1761; ristocetin 1237-1251, 1458-1471; and botrocetin 1277-1406. Adapted from (34,35). Sites for proteolytical processing are marked by a triangle.

facilitate the formation or rearrangement of interchain disulfide bonds by its intrinsic disulfide isomerase activity (33). The former was shown not to require the VWF-propeptide to be a contiguous part of the VWF protein to assist multimerization (31). The latter is supported by the identification of a transient disulfide-linked intermediate of the D1-D2 domains of the VWF-propeptide and the D'-D3 domains of the multimerization region of VWF (36). If indeed the VWF-propeptide would function as a catalyst in disulfide bond formation or in the rearrangement of existing disulfide bonds, this may explain how VWF is able to form HMW multimers in the acidic milieu of the Golgi.

Storage and secretion of VWF

The majority of VWF synthesized in the endothelium is secreted via the constitutive pathway and only a small amount *via* the regulated pathway (16).

Regulated secretion of ultra-large VWF multimers from specific endothelial storage organelles, Weibel-Palade bodies (37,38), occurs after activation of the endothelium. The Weibel-Palade bodies are rod shaped, striated organelles up to 4 µm in length and approximately 0.1 µm in width that release their content after fusing with the plasma membrane (39). Sorting of VWF to these storage organelles requires the presence of the VWF-propeptide (40), which is stored in the Weibel-Palade bodies at an equimolar ratio with mature VWF (41). After release from the storage organelle the VWF-propeptide is cleared four to five times more rapidly from the circulation than mature VWF, resulting in the non-equimolar ratio of VWF-propeptide over mature VWF observed in plasma (41,42). An additional source of regulated release of ultra-large VWF multimers (16) and VWF-propeptide (43) are platelets, which are derived from megakaryocytes and release VWF upon exocytosis of their α-granules.

Proteolysis of VWF by ADAMTS13

In normal plasma, HMW multimers of VWF are proteolytically processed to smaller fragments. This process is reflected by the triplet structure of a central band accompanied by two satellites, observed on VWF multimer gels (44) (Fig. 3). Recently the protease mediating this degradation of VWF was identified as ADAMTS13 (*A D*isintegrin *a*nd *M*etalloproteinase with *T*hrombo*s*pondin motifs) (reviewed by Plaimauer (45) and Porter (46)). VWF is cleaved by ADAMTS13 between residues Y1605-M1606, which are located in the A2 domain of VWF (Fig. 2) (47). A stretch of 73 aa in VWF, D1596 to R1668, was identified as the smallest region of VWF required for recognition and subsequent cleavage by ADAMTS13 under static conditions (48). Although the protease and its substrate are both present in the circulation, this does not result in the depletion of plasma of HMW multimers (49). Normally, the cleavage site in the A2 domain is inaccessible to ADAMTS13. It is exposed only after partial unfolding of VWF (49,50). *In vivo* this structural change is most likely induced by the shear stress exerted on VWF after binding to the endothelial surface; *in vitro,* denaturing agents such as urea or guanidine are able to make the cleavage site accessible (49,50). Padilla and co-workers proposed P-selectin as a candidate for securing extremely large VWF multimers to the endothelium, thereby facilitating the degradation of these multimers by ADAMTS13 (51).

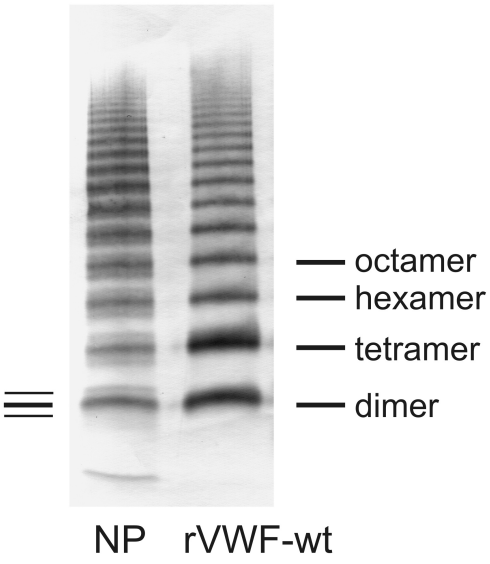

Fig. 3, multimer patterns of pooled normal plasma (NP) and recombinant wild-type VWF (rVWF-wt). The distribution of low, intermediate and high molecular weight multimers in normal VWF is shown. It reflects ADAMTS13 mediated proteolysis of VWF. Recombinant VWF does not show the triplet structure due to the absence of ADAMTS13 *in vitro*. The triplet structure of VWF is indicated on the left with a thick central line flanked by two thin satellite lines.

The average VWF:Ag level in plasma is approximately 10 µg/mL (10). However, a wide range in VWF:Ag levels is observed in healthy individuals, which is related to the addition of the ABO blood group antigens to VWF (52,53). Individuals with blood group O lack the glycosyltransferase needed to modify the precursor H antigen into A antigen (transferase A) or B antigen (transferase B) (54). VWF:Ag levels are 25-40% lower in blood group O carriers than in non-O blood group carriers (52,53). This variation may to some degree be explained by a protective effect of added carbohydrate groups on proteolytic degradation of VWF (55). Indeed, a protective effect of non-O compared with blood group O was found by Bowen in an *in vitro* test system (56). In their assay, blood group A showed a slightly higher protective effect towards ADAMTS13-mediated proteolysis than did blood group B (56). Both non-O blood groups were more protective than blood group O (56). Further removal of the terminal sugars on *N*-linked glycans is associated with more rapid cleavage of VWF by ADAMTS13, as was demonstrated for VWF lacking the H antigen (Bombay blood group) (57). The contribution of this rapid cleavage to the steady state plasma VWF level is

however uncertain as only slightly lower VWF:Ag levels were observed in individuals with Bombay blood group than in O blood group carriers (57). This suggests that apart from receptor-mediated clearance of VWF, increased susceptibility and possibly increased ADAMTS13 activity (58) may contribute to the lower levels of VWF:Ag observed in blood group O carriers.

A disturbed balance between secretion of ultra-large VWF multimers from the Weibel-Palade bodies and the proteolytic processing of VWF by ADAMTS13 may result in disease. Thrombocytic thrombocytopenic purpura (TTP), *e.g.*, may be caused by a lack of active ADAMTS13 in the circulation, due to mutations in the ADAMTS13 gene or autoantibodies, thus leading to the formation of microthrombi in the microvasculature of many organs (49,50). Another example is type 2A VWD, which is caused by increased ADAMTS13-mediated degradation of VWF due to mutations in the A2 domain of VWF (49).

Structure and functional regions of VWF

The use of proteolytic fragments of VWF, monoclonal antibodies and recombinant expression have resulted in the mapping of functional regions of VWF. These regions are located in specific domains in VWF (8,9), and are involved in the interaction with receptors on platelets, molecules in the extracellular matrix of the vasculature and exogenous molecules (Fig. 2). The domain binding to glycoprotein Ib (GPIb) on platelets is located in the A1 domain (34); the Arg-Gly-Asp (RGD) sequence that interacts with platelet receptor glycoprotein IIb/IIIa (GPIIb/IIIa) is located in the C1 domain (59-62); the collagen binding sequences are situated in the A1 and A3 domains (34); the D' and D3 repeats of VWF harbour the binding site of FVIII (63). This protein plays an important role in the fortification of the platelet plug by supporting localized coagulation resulting in fibrin formation. To date, no specific function has been assigned to the D4 domain or any of the B domains of VWF.

The non-covalent interaction observed between FVIII and VWF has been mapped to the first 272 amino acids of mature VWF (63) (Fig. 2). Proteolytic removal of the VWF-propeptide has been shown to be vital for the association and stabilization of FVIII by VWF (64). Complex formation with VWF makes FVIII less susceptible to phospholipid-dependent proteolysis by several proteases (65-70). VWF also shields FVIII from interaction with the low density lipoprotein

receptor-related protein and protects it from being internalized and targeted to the endosomal degradation pathway (71). Thus, low levels of VWF or a dysfunctional binding site for FVIII can result in reduced plasma FVIII activity. This ultimately translates to an increased risk for bleeding like in type 2N VWD (72).

VWF also contains binding sites for exogenous molecules. The ability of molecules as ristocetin and botrocetin to induce VWF-mediated aggregation of platelets is used in *in vitro* tests to analyze the functionality of VWF. The specific binding sites for these molecules are also indicated in Fig. 2 (34,35).

Considering the high percentage (8.2%) of cysteines in mature VWF, the presence of only six cysteines in the A1-A3 domains -corresponding to only 1%- is strikingly low. The A1 and A3 domains each form a large loop that is secured at the base by an intrachain disulfide bond between cysteines C1272-C1458 in the A1 domain and C1686-C1872 in the A3 domain (20). The A2 domain lacks this disulfide bond, which otherwise would render the Y1605-M1606 cleavage site for ADAMTS13 inaccessible (Fig. 2) (47).

The role of VWF in hemostasis

VWF has two important functions in blood coagulation. One is to mediate the formation of a platelet plug at a site of injury; the other is to function as a carrier of FVIII, preventing rapid clearance of FVIII from plasma. VWF functions as a molecular glue between the lining of the damaged blood vessel and circulating platelets passing by at high velocity, on the one hand, and between the activated platelets at the site of injury on the other hand. The adhesiveness of VWF is essential for the formation of a platelet plug under high shear conditions in the circulation. Other important factors are the presence of the platelet receptors GPIb and the integrin $\alpha_{IIb}\beta_3$ (GPIIb/IIIa).

An injury to the vasculature results in the exposure of subendothelial structures like collagen. VWF interacts with collagen *via* its A3 domain (73). Subsequently the binding site for the platelet receptor GPIb, located in the A1 domain of VWF, becomes accessible (74,75). The transient interaction of VWF with the platelet GPIb receptor decreases the velocity of the interacting platelets (76). The rolling of the platelets over the layer of VWF and additional interactions between collagen and the platelet receptors GPVI and $\alpha_2\beta_1$, leads to activation of

the platelets and subsequent expression of integrins such as GPIIb/IIIa on their surface. Firm attachment of the platelet is mediated *via* binding of GPIIb/IIIa to the RGD sequence in the C1 domain of VWF and other adhesive substrates containing this sequence such as fibrinogen and possibly fibronectin. Addition of second layer of platelets (aggregation) involves binding of VWF to the GPIb and GPIIb/IIIa platelet receptors and of fibrin to GPIIb/IIIa. This results in a layer of VWF and fibrin coating the adhered platelets which functions as a platform for circulating platelets and recruits them to the growing thrombus. The aggregation of platelets continues until the injury is sealed off and an unstable platelet plug has been formed. In the final stage this plug is reinforced by the formation of a stable fibrin network, which involves localized coagulation facilitated by the activated platelets and their secreted contents. The above described interactions have been reviewed by de Groot (77), Ruggeri (78), and Mendolicchio (75).

VWD

VWD is a common bleeding disorder (79). Most quantitative or qualitative deficiencies in the VWF protein result in mild bleeding from the mucosal areas observed as easy bruising or frequent nosebleeds. Normal procedures like dental extraction and tonsillectomy are, in VWD patients, often accompanied by extended bleeding. In women, VWD may result in heavy menses and extreme blood loss during delivery. Extremely low levels of VWF may also cause low FVIII activity leading to bleeding symptoms similar to those observed in hemophilia patients.

VWD is subdivided into three different types according to a simplified classification introduced by Sadler in 1994 (80). Qualitative VWF defects are designated type 2 VWD. Quantitative VWD is divided into partial deficiency (type 1) and severe deficiency (type 3) of VWF. Mutations causing quantitative VWD are spread throughout the entire VWF gene, which due to its size makes the identification of mutations causing the disease especially laborious. Quantitative VWF defects by definition only affect the level of the VWF protein in the circulation and do not influence the distribution between high, intermediate and low molecular weight multimers in plasma (80). Type 1 VWD is often dominantly inherited and is the most frequent of the VWD subtypes. Previously only a few mutations had been detected in type 1 VWD. This has however changed since the

start of the European Study "Molecular and Clinical Markers for the Diagnosis and Management of type 1 VWD" (MCMDM-1VWD) which revealed that 67% of the index cases have at least one candidate mutation (81). The majority of these mutations were missense mutations (81%) whereas a minority were mutations resulting in a null allele (12%) or affecting the promoter region of VWF (6%) (81). The rather uncommon type 3 VWD is inherited recessively and is characterized by the virtually complete absence of VWF antigen. In contrast to type 1 VWD, type 3 VWD is mostly caused by large deletions, nonsense mutations and frame shift mutations resulting in null alleles, even though missense mutations have been described as well (82). Type 2 VWD comprises four subtypes: 2A, 2B and 2M with altered interactions with platelets, and type 2N with decreased FVIII binding (80). Both types 2A and 2M are caused by decreased platelet-dependent functions. Type 2A is, however, associated with the absence of HMW multimers, while type 2M is not. Finally, type 2B exhibits increased affinity for GPIb. In contrast to quantitative VWF defects, mutations leading to qualitative defects are found mainly in certain functional domains of VWF, which facilitates the detection of the mutation.

The decreased ristocetin cofactor activity and decreased binding of VWF to collagen observed in type 2A VWD is a direct result of the lack of HMW multimers. Most of the underlying mutations are found in the A2 domain, while some reside in the A1 domain of VWF. They affect either biosynthesis and routing of the multimers (referred to as group 1 mutations; (83)) or increase the sensitivity to proteolysis by ADAMTS13 in plasma (referred to as group 2 mutations; (47,83,84)). The loss of HMW multimers and thrombocytes in blood of patients with type 2B VWD is explained by the increased affinity of VWF for GPIb (85). This results in spontaneous binding of VWF to platelets without previous activation, and the subsequent clearance of the platelet-VWF-complexes. The mutations are found in the A1 domain of VWF, which also contains the functional binding site for GPIb (86,87). In patients diagnosed with type 2M VWD amino acid residues in the A1 domain of VWF are mutated. These mutations result in decreased binding of VWF to GPIb but do not affect multimer assembly, as the multimer distribution in plasma is normal (87). In type 2N VWD mutations have been found in the D' and the D3 domains of VWF and correlate with normal multimerization but decreased FVIII binding of VWF (87). This is caused by alterations in the FVIII binding site

(63) or by the abrogation of VWF-propeptide cleavage, which has been shown to be required for optimal binding of FVIII to VWF (64).

Outline of this thesis

Studies of patients with varying severity of VWD and sequencing of the VWF gene has resulted in the identification of several missense mutations in VWF, among which mutated cysteine residues. The aim of the work described in this thesis was to evaluate how loss of cysteine residues located in different domains of VWF may cause quantitative VWF defects of different severity. Candidate missense mutations of cysteine residues detected in patients with quantitative VWD were studied to assess whether they indeed were the causative mutations. This was done by expression of full-length recombinant VWF in mammalian cells. The quantity of secreted and intracellular VWF as well as the quality of the produced VWF was studied.

In **Chapter 2a**, the effect on the level of dimerization and multimerization of two type 1 VWD mutations (C1130F and C1149R) was investigated and compared with the effects of three type 3 VWD mutations (C2671Y, C2739Y and C2754W). Further, we investigated whether the introduction of an alanine instead of a tyrosine at position 2671 would have an effect on recombinant VWF expression and these results are reported in **Chapter 2b**. The very low VWF antigen levels observed in patients with the C1130F, C1149R or C2671Y mutation were not completely reproduced *in vitro* (**Chapter 2a**). The hypothesis that increased clearance of the mutant protein may occur *in vivo* was tested. ADAMTS13-mediated proteolysis, assessment of the *in vivo* survival of the recombinant proteins in a murine model and the half-life of endogenous mutant VWF after infusion of DDAVP were investigated and showed increased clearance of all three mutant VWF proteins (**Chapter 3**).

In **Chapter 4**, we investigated a missense mutation of a cysteine residue that is neither located in the dimerization nor in the multimerization area of VWF. This mutation, C2362F, has been found in a subgroup of patients with autosomal recessive severe VWD having low VWF antigen levels, undetectable VWF ristocetin cofactor activity, but remarkably high FVIII coagulant activity. Expression of the C2362F mutation reproduced the severe VWD phenotype regarding the level of multimerized VWF and the quantity of VWF secreted.

However, a difference in the level of VWF degradation was observed between *in vitro* transfections and patients plasma indicating that the mutant protein may be sensitive to proteolytic cleavage *in vivo*, although this was not observed in the *in vitro* ADAMTS13-mediated assay.

During the investigation of the role of cysteine residues 2739 and 2754 in quantitative type 3 VWD (**Chapter 2a**), we observed that alterations of cysteine residues 2771 (88) and 2773 (89,90), which are also located in the CK domain of VWF, were reported to result in a different VWD phenotype, type 2A (the former subtype IID). We hypothesized that the difference in phenotype depends on whether the mutated cysteine residue is involved in the formation of an interchain bond or of an intrachain bond. To test our hypothesis we screened a family with type 2A(IID) VWD characteristics and detected a novel mutation, C2773S (**Chapter 5**). This residue is suggested to be involved in an interchain disulfide bridge in the CK domain (24) and therefore this finding supports our hypothesis that loss of interchain disulfide bonds in the CK domain results in type 2A(IID) VWD. In **Chapter 6** we investigated the possibility that a previously reported polymorphism, Y1584C, is associated with an increased risk for bleeding in patients treated with vitamin K antagonists. Unfortunately, the low frequency of this polymorphism and the small size of the study population did not allow a reliable estimate of its possible contribution to the risk of bleeding. The results obtained during these studies are summarized and discussed in **Chapter 7**.

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Dimerization and multimerization defects of von Willebrand factor due to mutated cysteine residues

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Abstract

In patients classified with type 1 and type 3 von Willebrand disease (VWD) missense mutations resulting in the loss of cysteine residues in the D3 domain (multimerization area) and in the carboxy-terminus (dimerization area) of the von Willebrand factor (VWF) have been identified. We have investigated how these structural changes result in a quantitative VWF deficiency and how they interfere with the dimerization and multimerization processes.

The effect of mutations in the multimerization area (C1130F, C1149R) and in the dimerization area (C2671Y, C2739Y, C2754W) of human recombinant VWF were investigated in transient transfection assays in 293T cells. All mutations resulted in reduced secretion of VWF in the medium and in intracellular retention. The amino-terminal mutants C1130F and C1149R showed impaired multimerization by lacking high molecular weight (HMW) multimers, in co-transfection experiments with wild-type VWF, the multimeric pattern was consistent with the pattern in the heterozygous type 1 VWD patients. The carboxy-terminal mutants C2739Y and C2754W showed strongly reduced to nearly absent secretion of VWF, consistent with type 3 VWD. The multimeric pattern of C2739Y and C2754W is characterized by the absence of HMW multimers, an excess of monomers and intervening odd-numbered multimeric bands, indicating a dimerization defect. The carboxy-terminal mutant C2671Y is different, with mildly reduced secretion, intermediate intracellular retention and a normal multimerization pattern.

We conclude that, in accordance with a phenotype of quantitative VWF deficiency, all cysteine mutants show impaired secretion, although the decrease of VWF *in vitro* appears lower than in the patients, suggesting additional, possibly heightened clearance, mechanisms *in vivo*.

Introduction

The von Willebrand factor (VWF) is a high molecular weight multimeric glycoprotein (0.5-10x10^6 Da) with adhesive properties. VWF mediates adhesion of platelets to the vessel wall and platelet-platelet aggregation. VWF forms a non-covalent complex with factor VIII. The unprocessed preproprotein of 2813 amino acids (aa) is targeted to the endoplasmic reticulum (ER) by the 22 aa long signal peptide. In the ER, the proVWF is glycosylated and the subunits are linked pairwise through covalent disulfide bonds at the carboxy-terminus. The proVWF dimers are further modified when passing through the ER and the Golgi apparatus. Multimers are formed from proVWF dimers by disulfide bonds at the amino-terminal end of the subunits. After formation of multimers, the VWF-propeptide is cleaved off, resulting in VWF multimers consisting of an even number of mature VWF subunits (2050 aa) (reviewed by Wagner (1) and Sadler (2)). The VWF protein contains a high number of cysteine residues (8.2%) all of which participate in forming intra- or interchain disulfide bonds (3).

Von Willebrand disease (VWD) is the most common inherited bleeding disorder. It is caused by dysfunctional VWF or by a deficiency of VWF. VWD is divided into three groups (4): type 1 refers to partial VWF deficiency, type 3 is characterized by an almost complete VWF deficiency and type 2 involves all functional defects of the VWF protein. The molecular basis of the disease has been elucidated for most type 2 VWD variants (5). The molecular basis of type 1 and type 3 VWD has been difficult to characterize, because mutations are not restricted to a specific region in the VWF gene.

Although type 1 and type 3 VWD are both characterized by a deficiency of VWF, the underlying genetic mechanisms appear to be different. Type 1 VWD has an autosomal dominant inheritance pattern, however recessive inheritance has also been described (6). Inheritance of type 3 VWD is autosomal recessive. The heterozygous carriers of type 3 mutations (mainly null alleles) usually have only mild or no bleeding symptoms and roughly 50% reduction of VWF levels. Therefore, these carriers of type 3 VWD are different from most type 1 VWD patients who have VWF levels well below 50%. We previously hypothesized that type 1 VWD could possibly be caused by missense mutations, resulting in a dominant negative defect, *i.e.,* mutant subunits interacting with normal subunits leading to a reduction of more than 50% of VWF levels. Following this hypothesis

we previously identified two missense mutations, C1130F and C1149R. These mutations, both occur in the D3 domain, which is involved in multimerization (7). C1149R has been shown to cause intracellular retention of VWF (7,8) (nomenclature of mutations according to reference (9)).

While mutated cysteines in the multimerization domain cause a dominant negative type 1 defect, we hypothesized that loss of cysteines in the dimerization domain (the 151 carboxy-terminal amino acid residues (10)) may also be responsible for quantitative defects. If mutant proVWF subunits are unable to form dimers, it is possible that only wild-type (wt) homodimers are formed (in the heterozygous state) and, if only these normal homodimers were transported to the Golgi, a reduction of VWF by 50% would be seen. This would mimic the effect of a null allele. Using this hypothesis, we have previously investigated VWD type 3 patients and identified a C2671Y mutation at the carboxy-terminus (11). Other C-terminally located cysteine mutations have also been identified in type 3 VWD patients (12-15).

To further investigate the VWF defect due to loss of cysteines in the multimerization and dimerization domains, we have expressed five different cysteine mutations; the amino-terminal C1130F and C1149R mutations, identified in type 1 VWD and the carboxy-terminal C2671Y, C2739Y and C2754W mutations, identified in type 3 VWD.

Materials and methods

Patients and mutations

The mutations we have expressed in transfections were originally identified in VWD patients. The C1130F and C1149R mutations were found previously in type 1 VWD patients, characterized by high penetrance of the phenotype and very low levels (0.10-0.15 IU/mL) of VWF antigen (VWF:Ag) (7). Two compound heterozygous type 3 VWD patients were described, one with a C2671Y mutation in combination with a deletion of the other allele (11), and one with a C2739Y mutation and an insertion of a cytosine (5221insC) on the other allele, leading to a premature stop codon (12). One type 3 VWD patient was homozygous for the C2754W mutation (13).

Plasmid construction

The pSVHVWF1 plasmid contained the full-length normal human cDNA of VWF cloned into the expression vector pSV7D (16) as previously described (17) and was kindly provided by Dr. Evan J. Sadler (Howard Hughes Medical Institute, St. Louis, MO, USA). The construction of mutant plasmid pSVHVWF-C1149R was previously described (7). The pSVHVWF-C1130F, C2671Y, C2739Y and C2754W plasmids were constructed *via* a general cloning strategy described in Fig. 1. The oligonucleotides used are listed in Table I. Restriction enzymes and T4 DNA ligase were from MBI Fermentas (St. Leon-Rot, Germany) or New England Biolabs (Leusden, the Netherlands). Oligonucleotides were synthesized on 0.2 µmol scale and purchased from Amersham Pharmacia Biotech (Roosendaal, the Netherlands) and were either PAGE (nrs 1 and 2) or HPLC purified. Construction of pSVHVWF-C2671Y and pSVHVWF-C2739Y required introduction of an *Sbf*I site in pSE280. The *Sbf*I site was introduced by annealing of oligonucleotides 1 and 2 (Table I) and ligation of the duplex in the *Pst*I and *Mlu*I digested pSE280 vector, yielding pSE280+SbfI, which was used for subcloning of the longer A-C wt VWF fragment via digestion with *Sbf*I and *Eco*RV. All constructs were transformed and propagated in *E. coli* DH5α. For transfection experiments DNA was purified using a plasmid maxi kit (Qiagen, Hilden, Germany). The DNA amount was quantified by measuring absorbance at 260 nm with an Ultrospec II spectrophotometer (Pharmacia LKB, Bromma, Sweden). DNA preparations used were screened for the respective mutations by restriction analysis.

Expression of recombinant VWF

293T human kidney cells (18) (kindly provided by Dr. J. Evan Sadler) were grown in Dulbecco's Modified Eagle Medium with 4.5 g/L glucose supplemented with 2 mM L-glutamine, 100 IU/mL penicillin, 100 IU/mL streptomycin and 10% (vol/vol) fetal bovine serum which were all from Gibco-BRL (Life Technologies, Paisley, United Kingdom). Cells were seeded to reach 50-70% confluence in T25 flasks (Becton Dickinson Labware, Franklin Lakes, NJ, USA) at transfection. 293T cells were transiently transfected using the calcium phosphate precipitation method (19). Cells were transfected for 15 hrs, washed twice, then overlaid with 5 mL Optimem 1 with Glutamax-1 (Gibco-BRL) supplemented with 0.5% human

serum albumin (CeAlb® for i.v. use, albumin 20%, CLB, Amsterdam, the Netherlands) and cultured at 37°C and 5% CO_2. After 21 hrs, when VWF production was still linear, conditioned media and cell lysates were collected. The protease inhibitor cocktail Complete™ with EDTA (Roche Diagnostics, Mannheim, Germany) was added to medium and cell lysate. Medium was centrifuged at 3000 rpm for 5 minutes at 4°C and supernatant was snap-frozen. The cells were rinsed with phosphate buffered saline and lysed with 0.8 mL Passive Lysis Buffer (PLB, Promega Corporation, Madison, WI, USA). Crude lysate was spun down at 10,000 rpm for 2 minutes at 4°C (Eppendorf Centrifuge model 5804R) and supernatant was snap-frozen.

In the single transfections a total of 10 µg wt or mutant construct was used per T25 flask. Titration series of co-transfections contained a total amount of 9 µg DNA. The molar amount of pSVHVWF promoter was corrected in each co-transfection by addition of the plasmid pSVHVWF-cDNA lacking the coding region of VWF. Furthermore, a pUC13 plasmid lacking promoter and multiple cloning site was used to bring the DNA amount to 9 µg. The pGL3 control plasmid harbouring the luciferase gene was included in all transfections to enable monitoring of the transfection efficiency. The luciferase activity in cell lysates was measured with a Luciferase Assay Substrate (Promega) using a luminometer (Lumat LB 9507, Berthold Technologies, Vilvoorde, Belgium). Similar transfection efficiencies were obtained for all the constructs in the single transfections. The luciferase activity was similar in both the single wt and co-transfection experiments that were performed in parallel (data not shown).

Analysis of recombinant VWF

Quantitative analysis of VWF

In conditioned medium and lysates the level of recombinant VWF (rVWF) was determined by a VWF:Ag Enzyme Linked Immunosorbent Assay (ELISA) as follows. A polyclonal rabbit anti-human VWF antibody (A082, Dako, Glostrup, Denmark) diluted 1000-fold in 0.1 M sodium-carbonate buffer (pH 9.4) was used to coat 96-well plates with flat-bottom wells (Greiner, Germany). Plates were coated overnight at 4˚C using 120 µL of the dilution per well and washed three times with PBS/0.1% Tween-20. Normal pooled plasma was diluted 20, 50, 100, 200, 400, 800,

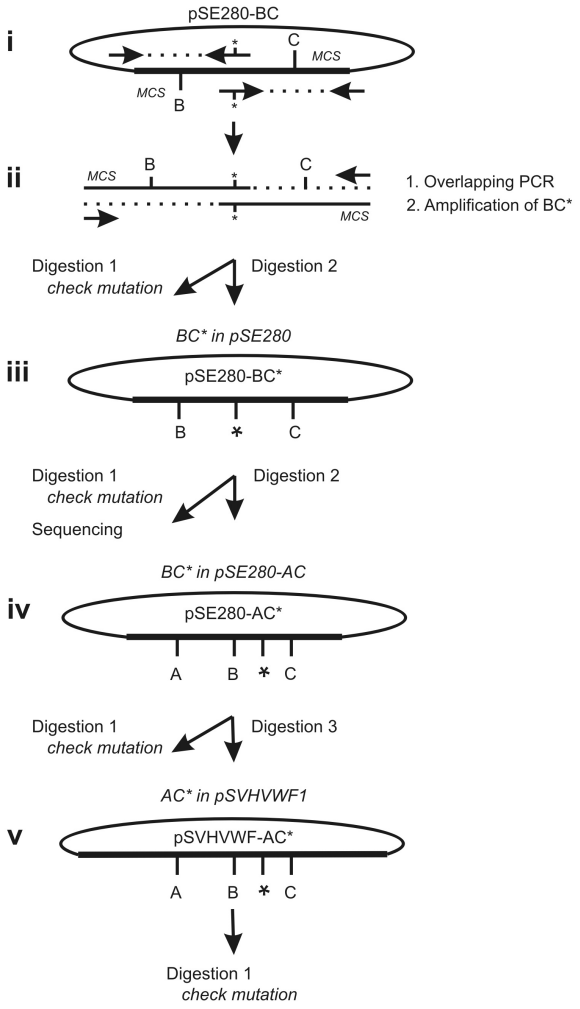

Fig. 1, schematic PCR and cloning schedule in pSE280 and pSVHVWF1. For explanation of abbreviations and digestion see Table I. **i**) Mutations were introduced in a short wt VWF B-C fragment inserted in pSE280 (Invitrogen, Leek, the Netherlands). Two separate partially overlapping mutant products were generated using a primer located in the multiple cloning site (MCS) of pSE280 and a mutagenic primer that introduced the appropriate nucleotide change (*). In addition, the latter primer also contained a silent nucleotide change to introduce a restriction site, as a marker for the presence of the mutation, digestion 1 (Table I). **ii**) The two overlapping fragments were pooled in a new PCR reaction, that was performed in absence of added primers to generate the full-length double stranded mutant fragment B-C*. Subsequently, the two vector primers were added to generate a sufficient amount of the mutant B-C* fragment for exchange with the wt B-C fragment that was cloned *via* digestion 2 (Table I) in pSE280. **iii**) Positive constructs were identified by digestion 1 and then both strands were sequenced using combinations of oligonucleotides 3-6 (Table I) and CEQ 2000 Dye Terminator Cycle Sequencing (Beckman Coulter, Fullerton, CA, USA). The mutant B-C* fragment was cloned *via* digestion 2 in pSE280 vector harbouring the longer wt VWF A-C fragment (Table I). **iv**) Introduction of the mutant B-C* fragment was verified by digestion 1. The mutant A-C* fragment was introduced in the pSVHVWF1 cDNA *via* digestion 3 (Table I). **v**) Constructs were tested for incorporation of the mutation by digestion 1.

Table I: Oligonucleotides used for introduction of mutations and for sequencing and enzymes for cloning

Construct [1]	Nb	F/R [2]	Sequence (5'-3') [3]	Position	Dig. 1 [6]	Frag. B-C [7]	Dig. 2 [8]	Frag. A-C [9]	Dig. 3 [10]
	1	F	p-GGTAGGTACTCGATA		*Sbf* I				
	2	R	p-CGCGTATCGAGTACCTACCTGCA	531-534, 551-555 [4]	*Sbf* I				
	3	F	TGCGCATGCTAGCTATAGTT	430-449 [4]					
	4	R	GGCTGAAAATCTTCTCTCATC	640-660 [4]					
	5	F	GACGTCGACCTGAGGTAAT	328-346 [4]					
	6	R	TTAACAACCGGTACCTCTA	451-469 [4]					
C1130F	7	F	CCAGAGCTtCGAGGAGAGG	3651-3669 [5]	*Taq*$^{\alpha}$I	3355-3878	*Sac*I, *Ngo*MIV	2505-3878	*Hind*III, *Ngo*MIV
C1130F	8	R	CCTCTCCTCGaAGCTCTGG	3651-3669 [5]	*Taq*$^{\alpha}$I	3355-3878	*Sac*I, *Ngo*MIV	2505-3878	*Hind*III, *Ngo*MIV
C2671Y	9	F	CTgCAGGATGGCTaTGATACTCAC	8269-8292 [5]	*Pst*I	8019-8508	*Sph*I, *Eco*RV	7357-8508	*Sbf*I, *Eco*RV
C2671Y	10	R	GTGAGTATCAtAGCCATCCTGcAGCGT	8266-8292 [5]	*Pst*I	8019-8508	*Sph*I, *Eco*RV	7357-8508	*Sbf*I, *Eco*RV
C2739Y	11	F	GTCAAGGTGGGAtcCTaTAAGTCTGAAGTA	8470-8499 [5]	*Bam*HI	8019-8508	*Sph*I, *Eco*RV	7357-8508	*Sbf*I, *Eco*RV
C2739Y	12	R	TACTTCAGACTTAtAGgaTCCCACCTTGAC	8470-8499 [5]	*Bam*HI	8019-8508	*Sph*I, *Eco*RV	7357-8508	*Sbf*I, *Eco*RV
C2754W	13	F	CAGGGCAAATGgGCCAGCAAAGC	8521-8543 [5]	*Hae*III			8508-9132	*Eco*RV, *Mun*I
C2754W	14	R	GCTTTGCTGGCcCATTTGCCCTG	8521-8543 [5]	*Hae*III			8508-9132	*Eco*RV, *Mun*I

[1] In pSVHVWF1. [2] Direction of oligonucleotide F:forward and R:reverse. [3] Nucleotide changes are in lower case, introduced restriction sites are underlined and (p) stands for 5' phosphorylation. [4] Position in pSE280. [5] Position in pSVHVWF1. [6] Restriction site introduced together with mutation. [7] Position of restriction sites in pSVHVWF1. [8] Restriction sites used for subcloning in pSE280 and pSE280-AC. [9] Position of restriction sites in pSVHVWF1. [10] Restriction sites used for exchange of mutant A-C* fragment into pSVHVWF1.

1600 and 3200-fold and used as standard. Conditioned medium was analyzed undiluted and 2, 4, 8, 16 and 32-fold diluted. Cell lysates were 8, 16, 32, 64, 128 and 256-fold diluted. Plasma standard and samples were diluted in sample buffer, PBS with 0.2% Bovine Serum Albumin (BSA, Fraction V, Sigma, Steinheim, Germany) and 0.1% Tween-20. 100 µL standard or sample was added to each well and incubated at room temperature (RT) for two hrs and washed three times. Polyclonal rabbit anti-human horseradish peroxidase (HRP) conjugated antibody (P226, Dako) was diluted 8000-fold in sample buffer. 100 µL of this dilution was added per well and incubated at RT for 2 hrs and washed three times. The substrate solution consisted of a 10 mg *O*-phenylene diamine (OPD, Sigma), 25 mL OPD-buffer (~100 mL 0.1 M Na_2HPO_4 and ~50 mL 0.1 M citric acid to pH 5.0) and 10 µL 30% H_2O_2 (Merck, Darmstadt, Germany). 100 µL substrate was added per well. The reaction was terminated after 20 minutes by addition of 100 µL 2 M H_2SO_4. The absorbance was read at 492 nm in a Organon Teknika Microwell System model 510 reader.

Qualitative analysis of VWF

VWF multimer analysis by non-reducing agarose gel electrophoresis with sodium dodecyl sulfate (SDS, Bio-Rad, Hercules, CA, USA) was essentially performed according to the protocol of Raines *et al.* (20). For all gels Sea-Kem® HGT (P) agarose (FMC Bioproducts, Rockland, ME, USA) was used. Samples were applied in wells in the 1.2% agarose cathode gel (cathode gel buffer: 0.1 M Tris, 0.15 M glycine and 0.1% SDS, pH not adjusted) and passed through the 0.8% agarose stacking gel (stacking gel buffer: 0.07 M Tris, 0.004 M EDTA and 0.4% SDS, pH 6.7). As the samples reached the separating gel, wells were emptied and filled with cathode gel. 1.5% agarose separating gel was used (separating gel buffer: 0.2 M Tris, 0.1 M glycine and 0.4% SDS, pH not adjusted). The gels were run horizontally for approximately 5 hrs in a 2117 multiphor II electrophoresis unit (LKB, Bromma, Sweden) with cooling at 14˚C and voltage at 150 V. VWF was transferred to Immobilon™-P PVDF membrane 0.45 µm (Millipore, Bedford, MA, USA) via capillary force overnight. For visualization of VWF multimers anti-human VWF A082 antibody (Dako) was used in combination with the peroxidase rabbit IgG ABC kit from Vectastain (PK-4001, Vector Laboratories Inc., Burlingame, CA, USA). PVDF membranes were stained with diaminobenzidine solution (10 mg 3,3'-diaminobenzidine (Sigma) in 100 mL PBS, to which 1 mL 3% $CoCl_2$ solution in

H_2O (Sigma), and 20 µL 30% H_2O_2 (Merck) were added). The reaction was terminated after 5-10 minutes by rinsing of the membranes in water.

Results

Single transfections

Three independent transfection experiments were performed in duplicate for all mutant and wt constructs. The mean relative expression of the mutant constructs as compared to wt in medium and lysate is shown as percentage of rVWF-wt (Fig. 2). The total amount of rVWF produced in wt, C1130F, C1149R, and C2671Y transfections was similar and higher than the total rVWF observed in

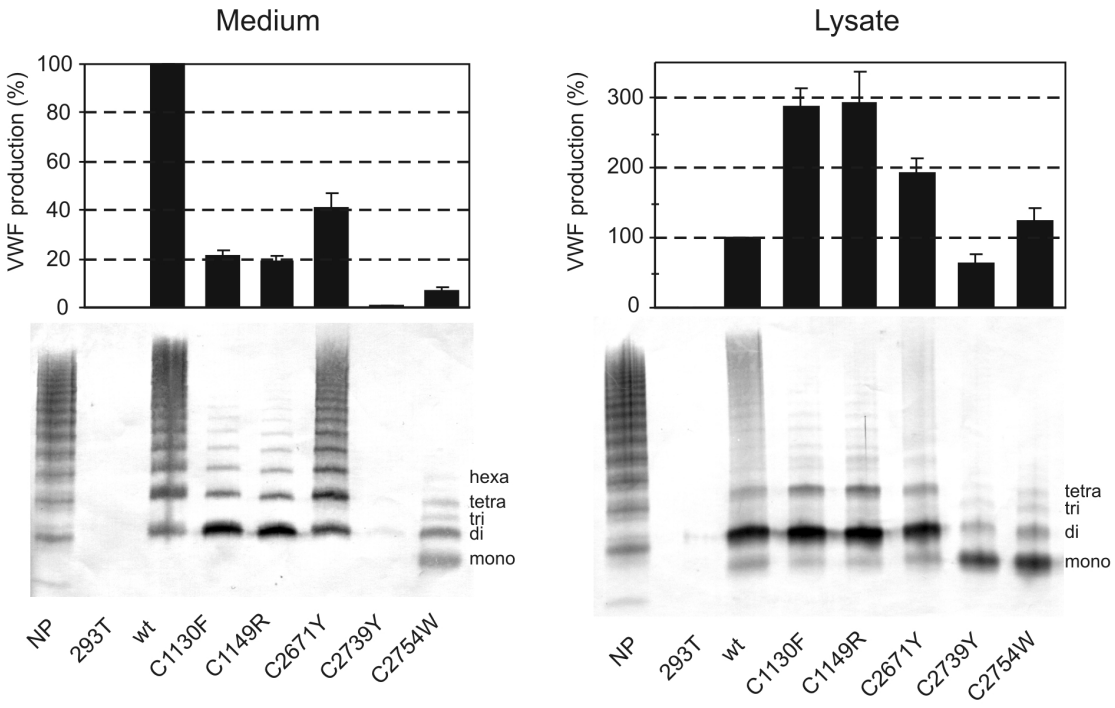

Fig. 2, transfections of wt or mutant pSVHVWF constructs in 293T cells. The VWF production in medium (left panel) and lysate (right panel) is expressed relative to the amount of wt protein. 100% of wt VWF corresponds to 0.37–0.56 µg/mL in conditioned medium and 1.0–1.8 µg/mL in cell lysate. Mean and standard deviation values are based on three independent experiments with duplicate transfections in each experiment. The corresponding multimeric patterns are shown in the lower panel. The construct names are indicated below each lane. 293T corresponds to untransfected 293T cells. Normal pooled plasma (NP) was used as reference. Oligomers are indicated.

transfections of C2739Y and C2754W, indicating rapid intracellular clearance of the C2739Y and C2754W proteins. C1130F and C1149R had a similar expression pattern, a considerable reduction in the secretion of rVWF in the medium (~20% of wt) and increased intracellular rVWF levels (~280% of wt). Furthermore, loss of high molecular weight multimers was seen. The carboxy-terminal mutant C2671Y differed from the other mutants in that it showed mildly decreased secretion of rVWF in the medium (~40%) and a normal multimeric pattern. The carboxy-terminal mutants, C2739Y and C2754W, were poorly secreted (~1% and ~8%, respectively). The levels in the lysate (~70% and ~125%, respectively) indicate that the mutant proteins were indeed expressed and that rVWF was retained in the cell. The multimer analysis of rVWF-C2754W in conditioned medium showed odd-number multimers (trimer), an excess of monomer and an absence of high molecular weight multimers. Secreted rVWF-C2739Y in medium was too low to be visualized by multimer analysis. The multimer patterns in cell lysates of both rVWF-C2739Y and rVWF-C2754W were similar, with an intervening band (trimer) and an excess of monomer.

Co-transfections

Co-transfections of wt and mutant constructs were performed with an increasing molar ratio of mutant over wt DNA, in order to study a possible interaction between mutant and wt VWF subunits. A basal level of 3 µg of wt construct was used, to which 1.5 µg, 3 µg or 6 µg mutant VWF construct was added. In corresponding wt transfections an increasing amount of wt VWF was added. Relative expression of co-transfections *versus* wt transfections was based on a duplicate transfection experiment (Figs. 3-7). The sum of rVWF in medium and lysate represents the total amount of rVWF. Overall, the total amount of rVWF produced in wt transfections increased linearly with the amount of DNA in the transfection. The level of rVWF-wt secreted was constant (about 70% of the total rVWF produced), independent of the amount of wt VWF plasmid used, indicating that the experimental cell system is not overloaded by 9 µg of DNA. The amount of rVWF observed in medium and lysate of co-transfections of all constructs seemed to follow an additive model (see C1130F).

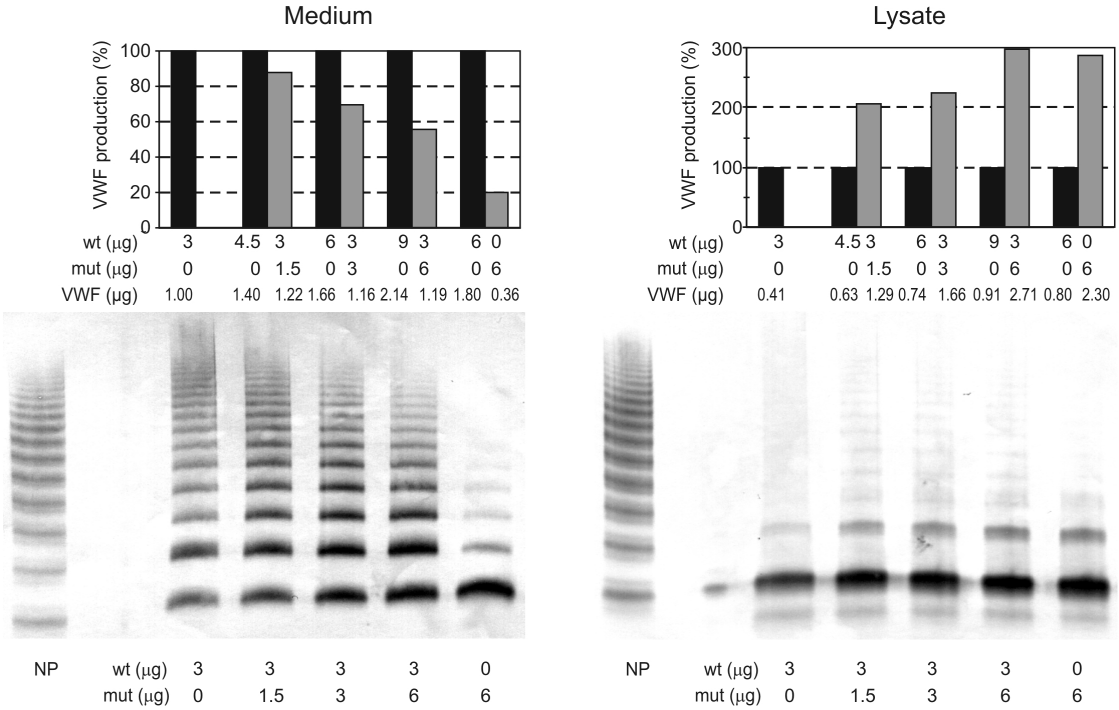

Fig. 3, co-transfection of wt and mutant pSVHVWF-C1130F showing the effect of increasing amounts of C1130F on VWF secretion and VWF multimer pattern. The VWF production in medium (left panel) and lysate (right panel) is expressed for each co-transfection (grey bars) relative to the amount of wt VWF of the corresponding wt transfection (black bars). The amount of wt and mutant (mut) construct added in each transfection is indicated below the bars as is the amount (µg) of VWF produced in either medium or lysate. Below the multimer blots the amounts of wt and mutant (mut) construct in the corresponding transfections are indicated. As reference, normal pooled plasma (NP) was used. Equal amounts of VWF were used in each lane, except for the transfection, where only mutant construct was expressed. The expression in medium was too low to apply an equal amount.

C1130F VWF and C1149R VWF

The total amount of rVWF produced in the co-transfections of wt with pSVHVWF-C1130F or pSVHVWF-C1149R and the corresponding wt transfections was similar, and increased with increasing amounts of transfected DNA (Figs. 3-4). However, the relative amount of rVWF secreted in the medium of co-transfections decreased compared to wt transfections. This decrease was dose-dependent, as was the relative increase of rVWF in the cell. These results indicate that the effect on secretion was solely caused by the mutant protein. For C1130F as a representative example of the additive model, we observed the following: 6 µg of

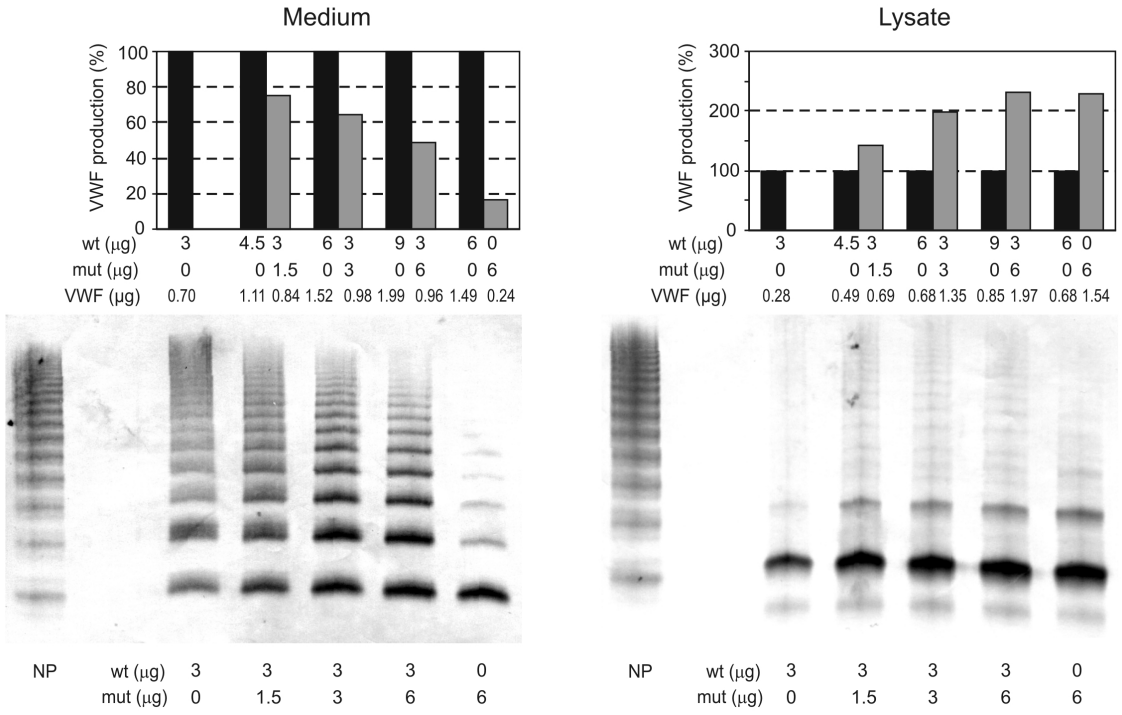

Fig. 4, co-transfections of wt and mutant pSVHVWF-C1149R showing the effect of increasing amounts of C1149R on VWF secretion and multimer pattern. The VWF production in medium (left panel) and lysate (right panel) is expressed for each co-transfection (grey bars) relative to the amount of wt VWF of the corresponding wt transfection (black bars). The amount of wt and mutant (mut) construct added in each transfection is indicated below the bars as is the amount (µg) of VWF produced in either medium or lysate. Below the multimer blots the amounts of wt and mutant (mut) construct in the corresponding transfections are indicated. As reference, normal pooled plasma (NP) was used. Equal amounts of VWF were used in each lane, except for the transfection, where only mutant construct was expressed. The expression in medium was too low to apply an equal amount.

wt construct alone resulted in 1.80 µg rVWF in the medium and 6 µg of C1130F construct resulted in 0.36 µg rVWF, predicting that in a co-transfection of 3 µg wt plus 3 µg mutant construct 1.08 µg ((1.80+0.36)/2) rVWF would be present in the medium, whereas 1.16 µg was actually observed (Fig. 3). In general, the secreted and intracellular amount of rVWF in the co-transfections, calculated from the 3 µg or 6 µg transfections of wt or mutant only, is in good agreement with the observed values. Interestingly, a mild decrease in the highest molecular weight multimers and a concomitant increase of the low molecular weight multimers, especially the dimer and tetramer, was observed. The multimeric pattern of the

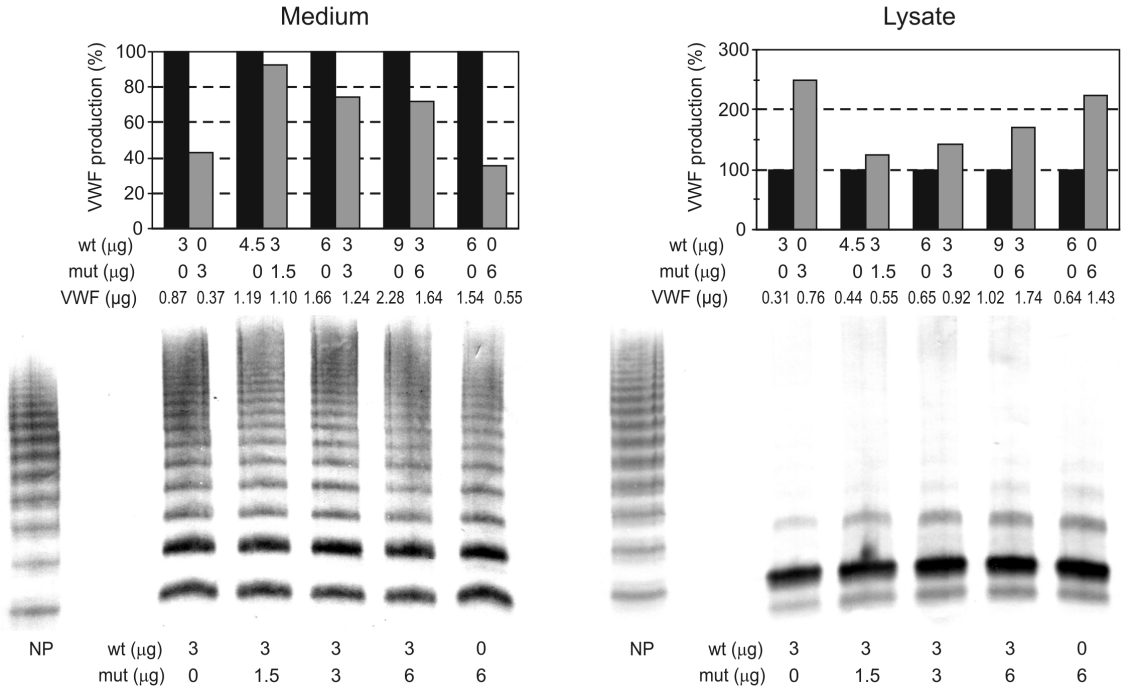

Fig. 5, co-transfections of wt and mutant pSVHVWF-C2671Y showing the effect of increasing amounts of C2671Y on VWF secretion and multimer pattern. The VWF production in medium (left panel) and lysate (right panel) is expressed for each co-transfection (grey bars) relative to the amount of wt VWF of the corresponding wt transfection (black bars). The amount of wt and mutant (mut) construct added in each transfection is indicated below the bars as is the amount (µg) of VWF produced in either medium or lysate. Below the multimer blots the amounts of wt and mutant (mut) construct in the corresponding transfections are indicated. As reference, normal pooled plasma (NP) was used. Equal amounts of VWF were used in each lane in the multimer analysis.

1:1 ratio co-transfection ("heterozygosity") was nearly normal and corresponds to the plasma VWF multimers in heterozygous type 1 VWD patients carrying these mutations (7).

C2671Y VWF

The results of the co-transfection of wt with pSVHVWF-C2671Y shown in Fig. 5 were similar to those obtained for the C1130F and C1149R constructs, except that the dose-dependent decrease of rVWF secreted in the medium and the increase of rVWF kept intracellularly were milder and that the multimeric pattern was normal in all co-transfections.

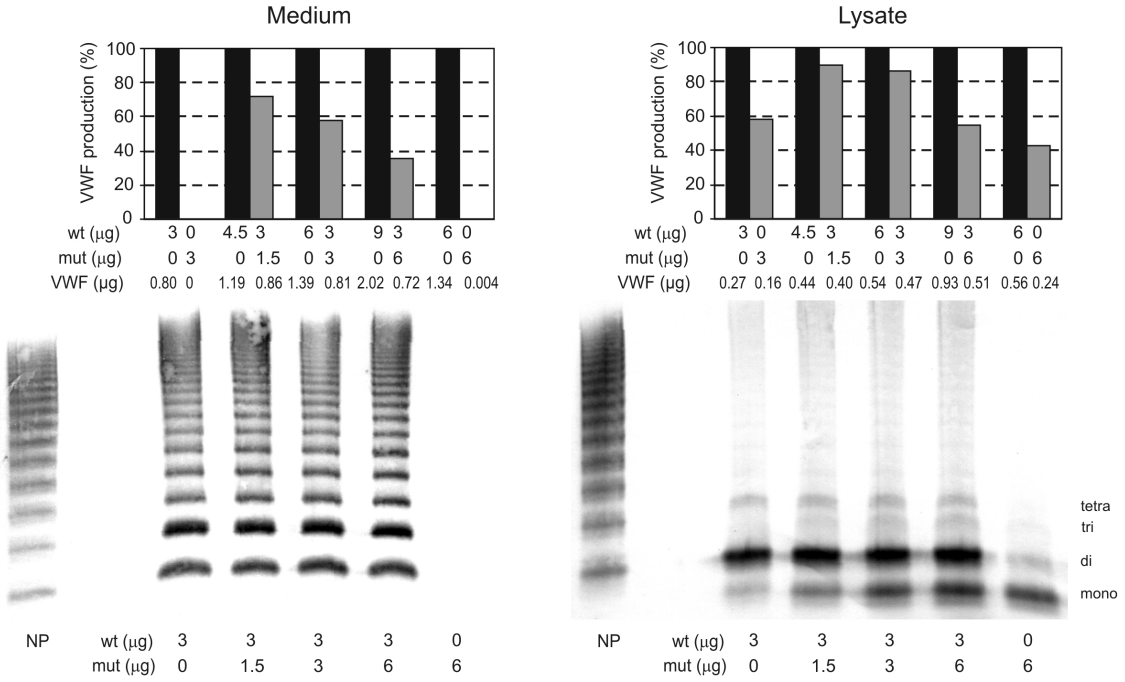

Fig. 6, co-transfections of wt and mutant pSVHVWF-C2739Y showing the effect of increasing amounts of C2739Y on VWF secretion and multimer pattern. The VWF production in medium (left panel) and lysate (right panel) is expressed for each co-transfection (grey bars) relative to the amount of wt VWF of the corresponding wt transfection (black bars). The amount of wt and mutant (mut) construct added in each transfection is indicated below the bars as is the amount (µg) of VWF produced in either medium or lysate. Below the multimer blots the amounts of wt and mutant (mut) construct in the corresponding transfections are indicated. As reference, normal pooled plasma (NP) was used. Equal amounts of VWF were used in each lane, except for the transfection, where only mutant construct was expressed. The expression in medium was too low to apply an equal amount. Oligomers are indicated.

C2739Y and C2754W VWF

C2739Y and C2754W showed a lower total amount of rVWF produced in the co-transfections compared to the corresponding wt-only transfections (Figs. 6-7). The total rVWF amount stayed at approximately 1.26 µg for C2739Y, while it rose slightly in co-transfections of C2754W as more construct was added. Titration of wt with C2739Y or C2754W caused a dose-dependent decrease of the rVWF secretion in the medium compared to wt transfections. The decrease of rVWF-C2739Y was not accompanied by the expected concomitant rise of the

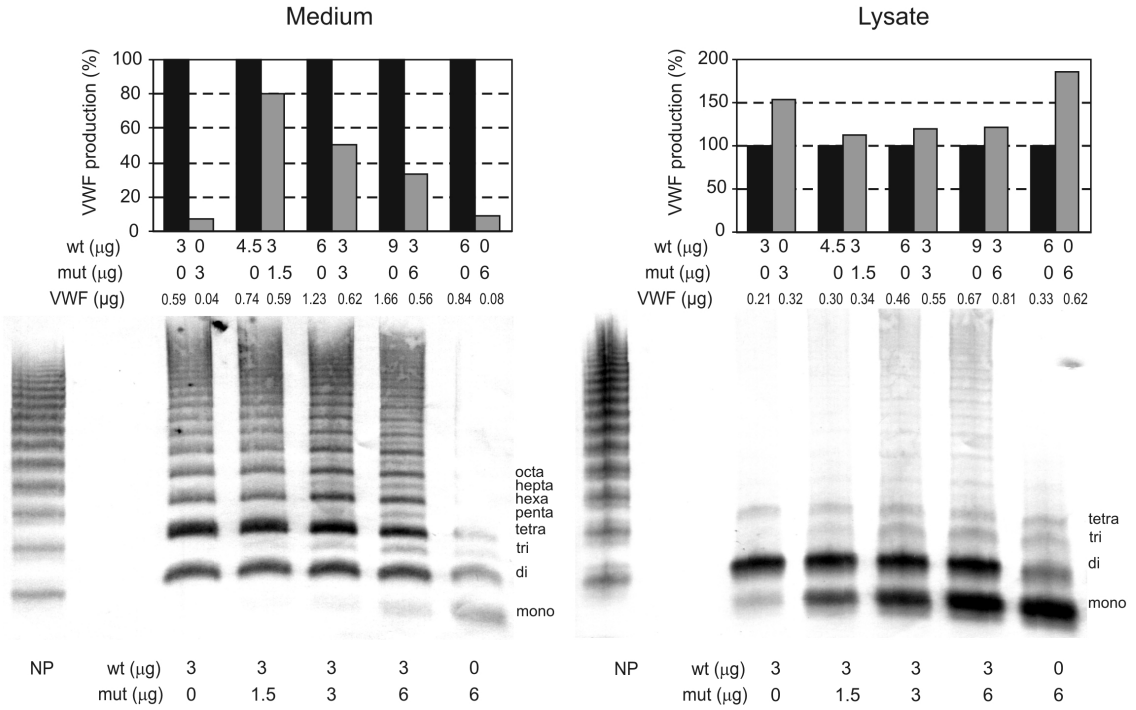

Fig. 7, co-transfections of wt and mutant pSVHVWF-C2754W showing the effect of increasing amounts of C2754W on VWF secretion and multimer pattern. The VWF production in medium (left panel) and lysate (right panel) is expressed for each co-transfection (grey bars) relative to the amount of wt VWF of the corresponding wt transfection (black bars). The amount of wt and mutant (mut) construct added in each transfection is indicated below the bars as is the amount (µg) of VWF produced in either medium or lysate. Below the multimer blots the amounts of wt and mutant (mut) construct in the corresponding transfections are indicated. As reference, normal pooled plasma (NP) was used. Equal amounts of VWF were used in each lane, except for the transfection, where only mutant construct was expressed. The expression in medium was too low to apply an equal amount. Oligomers are indicated.

intracellular level, on the contrary the level decreased, while the intracellular level of rVWF-C2754W, remained mildly elevated in all co-transfections. The formation of multimers of secreted rVWF did not seem to be affected by the increasing ratio of mutant over wt in the co-transfection of C2739Y, whereas intervening odd-numbered multimers were formed throughout the whole spectrum of multimers for rVWF-C2754W. In the lysate of the co-transfected cells monomer and dimer forms of rVWF are the most pronounced entities, for both C2739Y and C2754W. The odd-numbered VWF multimer, a trimer, is noticeable, indicating addition of mutant monomers to otherwise normal dimers.

Discussion

We have investigated the effect of the loss of cysteine residues, involved in either dimerization or multimerization, on the quantity and quality of the VWF produced. The two mutations in the D3 domain, C1130F and C1149R, were identified in type 1 VWD patients. Their phenotypes are characterized by a dominant inheritance pattern with high penetrance and VWF antigen levels between 0.10-0.15 IU/mL. We postulated that the strikingly low levels of VWF:Ag could be explained by the interaction of the mutant subunit with the normal subunit, resulting in retention of normal subunits in the ER and the Golgi, causing a dominant negative defect. We have previously reported that the mutation C1149R decreases the secretion of co-expressed normal VWF and causes intracellular retention and degradation of VWF (7,8). In this study we performed expression studies of both C1149R and C1130F. Similar results were obtained for both mutations (Figs. 2-4): impaired secretion (~20% of wt) and intracellular retention (~280% of wt) of rVWF. In the 1:1 ratio (wt:mutant) co-transfection, mimicking the heterozygous type 1 patients, secretion of VWF was 69% and 63% for C1130F and C1149R, respectively, and both co-transfections demonstrated dose-dependent intracellular retention of rVWF. We have demonstrated that the reduced secretion is caused by intracellular retention and not by reduced expression of the mutant construct. Both in the present and in the previous study of C1149R (7) we did not find the decrease of VWF that is observed in the plasma of heterozygous patients with C1130F or C1149R. The more pronounced decrease of plasma VWF in the patients could be explained by mechanisms not reflected by the 293T cell system, such as an increased clearance of mutant VWF by VWF cleaving protease and/or regulated secretion.

Both C1130F and C1149R rVWF show abnormal multimeric patterns in co-transfections (Figs. 3-4), illustrating a dominant negative effect at the qualitative level. However, the quantitative results support an additive rather than a dominant negative model as the observed amounts of rVWF secreted or retained can be predicted by the summation of single transfections of wt or mutant constructs alone. Bodo *et al.* (8) showed proteosomal degradation of intracellularly retained C1149R in Fur4BHK cells with stable expression of C1149R. This degradation could explain the dominant negative effect of the mutation C1149R observed in patients. Our 293T cell system may not reflect this dominant

negative aspect due to the transient nature of the transfection and the collection of medium at a time when production of VWF was still linear. In the linear phase the contribution of intracellular degradation may be negligible.

The intracellular retention and impaired multimerization of both C1130F and C1149R are probably due to disturbed protein folding. The decrease of secreted rVWF-C1149R was previously shown to not be due to the effect of the resulting unpaired cysteine 1169, which normally pairs with C1149. It was shown that expression of the double mutant C1149R + C1169S did not restore the defect of C1149R alone (8). Another argument for a conformational change is the fact that cysteine 1130 and 1149 are likely to form intrachain bonds (3) and are not directly involved in the intersubunit bonds required for multimerization. Dong *et al.* (21) demonstrated that even mutating the three cysteines (C1222, C1225, C1227) involved in the formation of intersubunit disulfide bonds did not influence assembly or secretion of VWF, indicating that the conformation of VWF as determined by intrachain bonds is more important for normal multimerization than the individual interchain bonds. Finally, C1130F and C1149R involve mutations to bulky or charged amino acids, predicting major effects on the conformation.

We have also investigated mutated carboxy-terminal cysteines that may be involved in dimerization. Three mutated cysteine residues (C2671Y, C2739Y and C2754W), all described in type 3 VWD patients (one reported by us (11) and two by others (12,13)), were studied. Most mutations described in type 3 VWD are null alleles (22). We hypothesized that these mutated cysteines at the carboxy-terminus could mimic null alleles when the mutated monomer is not able to dimerize, when it is retained and degraded in the ER and, when subsequently, only homodimers from the normal allele are routed to the Golgi and secreted. This would result in a level of about 50% normal VWF (in a heterozygous carrier of such a mutation), which is the same as in carriers of a null allele. In recombinant expression a striking difference in rVWF levels and multimer patterns was observed for C2671Y compared to the two other carboxy-terminal mutants. C2671Y showed completely normal dimerization and multimerization (Figs. 2 and 5). The apparent lack of influence of C2671Y on dimerization may be explained by its location. C2671 lies within the 151 carboxy-terminal amino acids that have been demonstrated by Voorberg *et al.* (10) to be required, and sufficient, for dimerization. C2671 was therefore anticipated to be important for dimerization.

However, only the carboxy-terminal 90 amino acids are homologous to the cystine knot (CK) family of proteins (23). Therefore, it is possible that only amino acids 2724-2813, excluding C2671, are essential for dimerization. The VWF:Ag level in the patient carrying the C2671Y mutation in compound heterozygosity with a complete VWF gene deletion, corresponding to homozygosity for C2671Y, is only 2%, whereas the expression in medium of 293T cells is 40% of wt. This discrepancy can be explained by increased physiologic proteolysis of VWF C2671Y, that was demonstrated in this patient's plasma (24), but which is lacking in the medium of the *in vitro* expression system. Our results suggest that the mutation C2671Y interferes with intracellular routing, has little or no influence on dimerization and is possibly more sensitive to proteolytic degradation in plasma.

The missense mutations C2739Y and C2754W were shown to interfere with dimerization. Excess of mainly intracellular monomers and odd-numbered multimers was found for both mutations (Figs. 2 and 6-7). This indicates N-terminal pairing of mutant monomers in the Golgi. The pattern with odd-numbered multimers in the heterozygous transfection of C2754W was previously described by Schneppenheim *et al.* and demonstrated in plasma of a heterozygous carrier (13). Odd-numbered multimers were not observed in medium of C2739Y co-transfections. The difference in secreted multimers of C2739Y and C2754W may lie in the survival of the mutant monomers in the cell. It is unlikely that a missense mutation affects translation; we therefore assume that the mutant monomer is misfolded, is retained in the cell and cleared at a high rate as has previously been described (10). The linear increase in the total amount VWF in C2754W co-transfections compared to the fixed low value in C2739Y co-transfections indicate that the C2739Y mutation causes a more profound conformational change than the C2754W. This would explain the different rate of clearance and the impossibility for C2739Y to pass the ER and participate in multimerization in the Golgi, whereas C2754W may pass the ER.

Both rVWF-C2739Y and rVWF-C2754W were retained in the cell and secreted at very low levels in medium, 1% and 8%, respectively (Fig. 2), which corresponds to a type 3 phenotype. Further, the co-transfection experiments of mutant and wt in a 1:1 ratio showed about 50% rVWF secretion for C2739Y and C2754W, which agrees with the phenotype of a heterozygous carrier of type 3 VWD. However, heterozygosity for mutations of some cysteine residues in the CK domain results in type 2A (formerly subtype IID) VWD. Expression studies have shown a similar

dimerization defect (13,25) as in the mutants in this study. This apparent phenotypic discrepancy may be explained by differences in the involvement of cysteines in either intrachain or interchain disulfide bonds. All mutations that have been described in association with a type 3 phenotype, C2739Y (12), C2754W (13), C2804Y (14) and C2806R (15) are involved in intrachain disulfide bonds (23), whereas the mutations associated with a 2A(IID) phenotype involve interchain disulfide bonds: C2771Y (25), C2771S (25) and C2773R (13,26). We hypothesize that the loss of a cysteine involved in an intrachain disulfide bond considerably disturbs the conformation of the VWF subunit, leading to hampered dimerization and rapid degradation in the ER. The amount of abnormal monomer contributing to the total amount of VWF would be small and would not cause a dominant negative effect. This would result in a quantitative defect with little influence on multimeric structure, *i.e.,* a type 3 phenotype in homozygotes and only 50% reduction of VWF in heterozygotes, mimicking the effect of null alleles. On the contrary, the mutations interfering with interchain bonds do not disrupt the conformation of the monomer, but do reduce the covalent dimerization of the subunit (23). As the conformation of the monomer is not dramatically affected, it is possible that a higher fraction of monomers escape degradation in the ER and is routed to the Golgi. Since the proportion of mutant monomer to normal monomer is higher in this case, the multimerization will be significantly influenced by N-terminal disulfide bonding of the excess mutant monomers resulting in a type 2A(IID) VWD multimer pattern.

In conclusion, we have shown that the cysteine mutations investigated in this study cause quantitative VWF deficiency although the effect is not as strong as that observed in patients. The discrepancy in VWF levels of C1130F, C1149R and C2671Y observed *in vitro versus in vivo* may be explained by physiological factors such as heightened clearance. The loss of cysteines 2739 and 2754 corresponds with the type 3 VWD phenotype and the apparent lack of influence of the C2671Y mutation on dimerization is most likely due to its location just outside the conserved CK domain.

Acknowledgements

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Intracellular retention of von Willebrand factor C2671Y is due to the loss of the disulfide bond and not to the introduction of the bulky side chain of the tyrosine residue

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Abstract

The replacement of cysteine 2671 by tyrosine, C2671Y, in von Willebrand factor (VWF) was previously identified in a patient with type 3 von Willebrand disease (VWD). This naturally occurring mutation was transiently expressed in 293T cells. C2671Y was compared with the alanine variant, C2671A, with respect to secretion, retention and multimerization. We assessed whether the retention of the mutant protein is caused by loss of the cysteine and its disulfide bond or is the result of a combined effect of the lost disulfide bond and the introduced bulky side chain of the tyrosine residue. Expression of the C2671A and C2671Y constructs resulted in slightly higher levels of recombinant VWF (rVWF) C2671A than of rVWF-C2671Y in the conditioned media of transfected cells. Intracellular levels of rVWF-C2671Y were slightly higher than observed for rVWF-C2671A. Expression of wt, C2671A and C2671Y rVWF resulted in normal multimerization of rVWF both in single transfections and in co-transfection experiments. These results indicate that retention of VWF-C2671Y is predominantly caused by the loss of the cysteine residue and the corresponding disulfide bridge and not by the introduction of the bulky and hydrophobic side chain of the tyrosine residue.

Introduction

In a previous study (1), mutations of cysteine residues located in both the multimerization and dimerization area of von Willebrand factor (VWF) were studied. Mutations of cysteines C1130, C1149, C2671, C2739 and C2754 were all identified in patients with a predominantly quantitative defect of VWF (2-5). Interestingly, examination of the human VWF database (http://www.sheffield.ac.uk/vwf) revealed that most mutated cysteines in VWF were altered to a bulky aromatic or charged amino acid residue. Of the 27 cysteine mutations reported, one was a stop codon, four were altered to serine or glycine and as many as 22 represented alterations to tryptophane, tyrosine, phenylalanine or arginine. The observation that cysteine residues were mutated to bulky or charged amino acids in 81% of the cases made us hypothesize that the change to such an amino acid may have additional effects on the conformation of the VWF subunit and thus influence the biosynthesis and secretion of VWF and result in a more severe phenotype.

To investigate this hypothesis we compared the recombinant expression of the naturally occurring C2671Y mutation with the C2671A mutation. If the side chain of the tyrosine residue would introduce disadvantageous conformational changes in the VWF subunit, then the secretion levels of VWF-C2671A are anticipated to be less affected than those of VWF-C2671Y.

Materials and Methods

Plasmid construction

Construction of plasmids pSVHVWF encoding VWF-wt and VWF-C2671Y was described previously (1). The C2671A VWF mutation was introduced in the pSVHVWF plasmid containing the full-length cDNA of wt-VWF. Oligonucleotides 1 and 2 (Table I) were used in combination with the QuikChange® XL site-directed mutagenesis kit from Stratagene (La Jolla, CA, USA) to introduce the C2671A mutation in pSVHVWF using the following cycling conditions: (1x 95°C 60 s, 18x (95°C 50 s, 60°C 50 s and 68°C 24 minutes), 1x 68°C 7 minutes). The presence of the mutation was confirmed by restriction digestion of plasmid DNA with *Hae*II. Subsequently, pSVHVWF-C2671A DNA was directly sequenced using the

Table I: Oligonucleotides for mutagenesis and sequencing

Oligonucleotide #	Direction	Sequence* (5' to 3')	Position†	Purpose§
1	Forward	CGCTCCAGGATGGCgcTGATACTCACTTCTGC	8267-8298	Mutagenesis
2	Reverse	GCAGAAGTGAGTATCAgcGCCATCCTGGAGCG	8298-8267	Mutagenesis
3	Forward	CACCGACATGGAGGATGCCG	7629-7648	Sequencing
4	Reverse	GCACATCGTTGATGTCAATGG	8572-8552	Sequencing

*Nucleotide substitutions indicated in lower case, the additional *Hae*II restriction site introduced in pSVHVWF-C2671A is underlined. †Nts in pSVHVWF (1). §Mutagenesis, introduction of C2671A in pSVHVWF; Sequencing, sequencing of plasmid.

CEQ 2000™ Dye Terminator Cycle Sequencing Kit (Beckman Coulter, Fullerton, CA, USA) in combination with oligonucleotides 3 and 4 (Table I). The mutant *Psh*AI-*Eco*RV C2671A VWF fragment replaced the wt fragment in pSVHVWF, creating pSVHVWF-C2671A. The constructs were transformed and propagated in *E. coli* DH5α. DNA used in transient transfection experiments was purified with the plasmid maxi kit from Qiagen (Hilden, Germany) and the preparations used were screened by restriction with *Hae*II as described.

Expression of recombinant VWF

Single transfections of wt and mutant pSVHVWF-C2671A and pSVHVWF-C2671Y plasmids and co-transfection of wt and pSVHVWF-C2671A constructs were performed according to the calcium phosphate precipitation method in 293T human kidney cells. The transfections and the collection of conditioned medium and cell lysate and subsequent quantitative and qualitative analysis were as previously described (1).

Results

Single transfections

The effect of the C2671A mutation on dimerization, multimerization and on levels of VWF in conditioned medium of 293T cells was studied in transient single

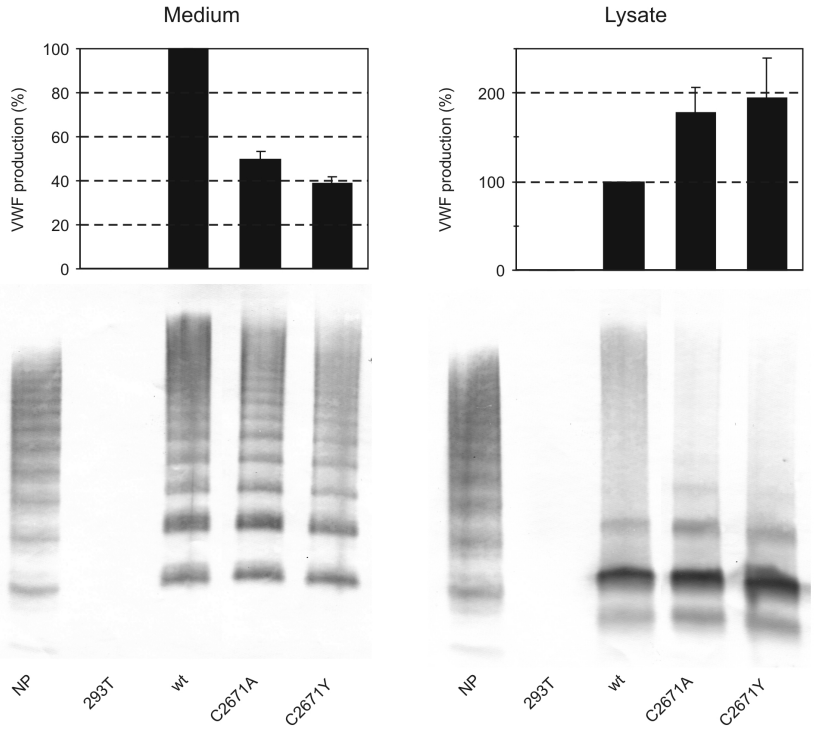

Fig. 1, single transfections of wt and mutant pSVHVWF constructs in 293T cells. The VWF production in medium (left panel) and lysate (right panel) is expressed relative to the amount of wt VWF protein. One hundred percent of wt VWF corresponds to 0.24-0.34 µg/mL in conditioned medium (5 mL) and 0.95-1.1 µg/mL in cell lysate (0.8 mL). Mean and standard deviation are based on six measurements (three independent experiments with two transfections in each experiment). The corresponding multimeric patterns are shown in the lower panel. The construct names are indicated below each lane. 293T corresponds to untransfected 293T cells. Normal plasma (NP) was used as reference.

transfections and co-transfections of wt and mutant constructs. Three independent single transfections including duplicate transfections of each construct were performed. The average secretion of recombinant VWF (rVWF) in conditioned medium was 49% and 39% of rVWF-wt for rVWF-C2671A and rVWF-C2671Y, respectively (Fig. 1). The intracellular VWF levels were 178% for rVWF-C2671A and 194% for rVWF-C2671Y compared to rVWF-wt. Total VWF, *i.e.,* the sum of VWF secreted in medium and VWF in the cell lysate, were 2.4 µg, 2.2 µg and 2.2 µg for rVWF-wt, rVWF-C2671A and rVWF-C2671Y VWF, respectively. This indicated that the reduced levels of mutant VWF found in the conditioned media were not caused by a lower production of the mutant protein. The relative amounts of rVWF-C2671Y secreted and retained were in accordance with results

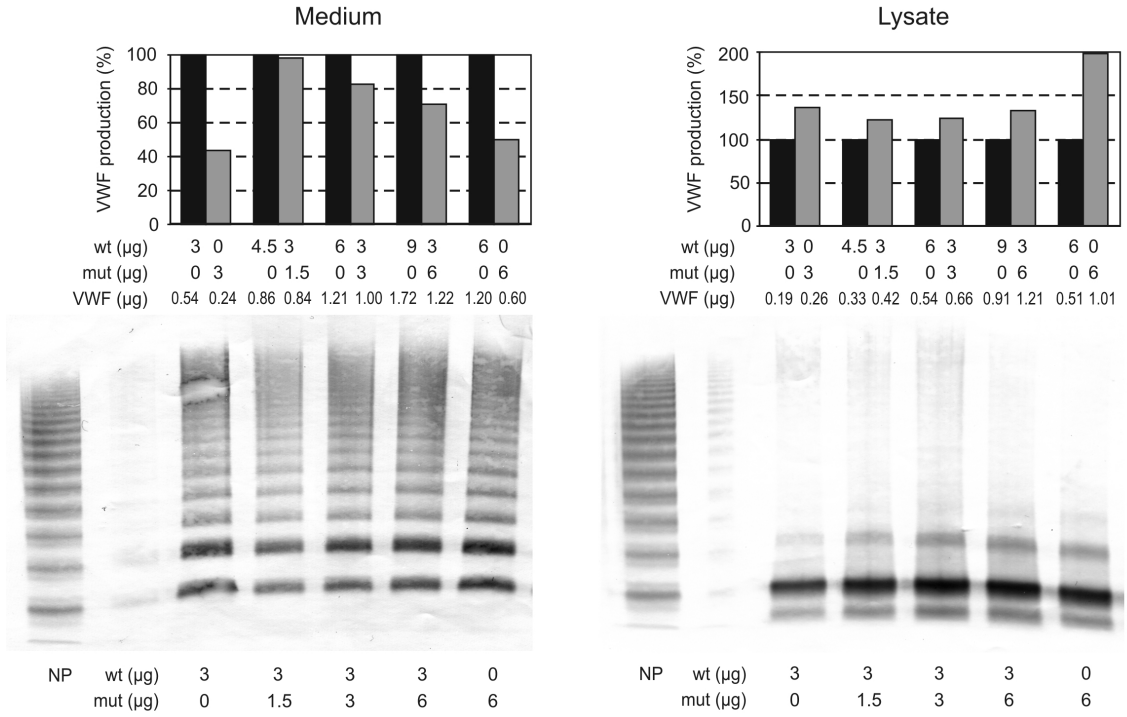

Fig. 2, co-transfections of wt and mutant C2671A pSVHVWF constructs. The VWF production in medium (left panel) and lysate (right panel) is expressed for each co-transfection (grey bars) relative to the amount of wt-VWF of the corresponding wt transfection (black bars). The amount of wt and mutant (mut) construct added in each transfection is indicated below the bars as is the amount (µg) of VWF produced in either medium or lysate. Below the multimer blots the amounts of wt and mutant (mut) construct in the corresponding transfections are indicated. As reference, normal plasma (NP) was used. Equal amounts of VWF were applied in each lane.

obtained previously (Chapter 2a) (1). Although the difference in secretion between rVWF-C2671A and rVWF-C2671Y was only small it was statistically significant ($p<0.005$, two-tailed students t-test). However, the intracellular levels of rVWF-C2671A and rVWF-C2671Y were not significantly different ($p=0.5$, two-tailed students t-test). Normal multimerization of VWF was observed for both rVWF-C2671A and rVWF-C2671Y (Fig. 1).

Co-transfections

In co-transfections, 3 µg wt construct was supplemented with 1.5, 3, or 6 µg of mutant or wt construct. Previously, we have shown that addition of up to 9 µg of

DNA leads to a linear increase in VWF production in this cell system (1). The total amount of VWF produced in co-transfections of C2671A and wt constructs was approximately equal to that found in the corresponding wt transfections (Fig. 2). A relative decrease from 98% to 71% of secreted rVWF, and a slight increase from 122% to 133% of retained rVWF was detected as the amount of C2671A plasmid was increased from 1.5 µg to 6 µg. As observed in the single transfections, the multimerization of rVWF-C2671A appeared normal and rVWF-C2671A did not seem to impair the multimerization in co-transfections (Figs. 1-2). These results were similar to those obtained in co-transfections of wt and C2671Y construct ((1), see Fig. 5, Chapter 2a).

Discussion

We have previously identified the C2671Y VWF mutation in a patient with type 3 VWD (3). The patient was found to be compound heterozygous for the C2671Y mutation and a deletion of the other allele. In the present study we examined whether the introduction of a residue with an aromatic side chain like tyrosine would have a disadvantageous effect on the level of recombinant VWF secreted additional to the one created by the loss of the cysteine bond. Therefore, we compared the expression of rVWF-C2671Y, rVWF-C2671A and rVWF-wt in 293T cells. The total VWF production, *i.e.*, VWF in medium and lysate, was similar for the rVWF-C2671A and rVWF-C2671Y variants (Fig. 1). The alanine variant seemed to be secreted into the medium slightly better and less retained intracellularly. However, the difference in retention was not statistically significant. Furthermore, the levels of secreted VWF in co-transfections of wt and C2671A VWF constructs were similar to those in the co-transfections of wt and C2671Y VWF plasmids (Fig. 2 and Chapter 2a or reference (1)).

On the basis of these results, we conclude that the retention of VWF-C2671Y is primarily caused by the loss of the cysteine and its disulfide bond, and that the contribution of the size of the introduced aromatic side chain is only minimal. On the one hand, we observed that the number of cysteines mutated to a serine or a stop codon in patients with VWD is clearly lower than the expected number, suggesting that these alterations may not always lead to disease (Table II). On the other hand, the number of cysteines substituted by phenylalanine, arginine and tryptophane observed in patients is higher than expected, which suggests that

Table II: Distribution of mutated cysteines in VWF

Amino acid		Codons*	Alteration	Theoretical distribution (%)†	Observed number§	Expected number**
Small	Ser	AGY/TCN	Transversion	19.3	2	5.2
Small	Gly	GGN	Transversion	9.7	2	2.6
Bulky	Phe	TTY	Transversion	9.7	6	2.6
Bulky	Trp	TGG	Transversion	9.7	4	2.6
Bulky	Arg	CGN	Transition	21.0	8	5.7
Bulky	Tyr	TAY	Transition	21.0	4	5.7
Nonsense	Stop	TGA	Transversion	9.7	1	2.6
Total				100.0	27	27.0

*Possible single nucleotide changes from a cysteine-encoding TGC- or TGT-codon. The altered nucleotide is underlined. Note that there are two changes that lead to a serine codon. Y = C or T. N = any nucleotide. †Distribution based on the alteration of the first, second or third position of the cysteine codons and a transition to transversion ratio of 2.17 for nonsynonymous codon changes according to Freudenberg-Hua *et al.* (6). §Number of different cysteine positions altered. Data were compiled from http://www.sheffield.ac.uk/vwf. **Expected frequency based on theoretical distribution where synonymous mutations were excluded.

these large residues more often cause VWD. The virtual absence of smaller amino acids at these positions supports this hypothesis.

It is not surprising that only one mutation to a stop codon has been reported since it would result in a null allele, which in a heterozygous carrier would result in only mildly reduced VWF:Ag levels. To result in VWD, an additional mutation would be required. Likewise, no silent mutations of cysteines are reported in the database. Finally, it may be that the effect of the mutation is also dependent on the position and function of the cysteine residue in the VWF protein. It would therefore be interesting to compare phenylalanine, arginine and tryptophane *versus* serine and glycine at the positions of other mutated cysteines in VWF.

Acknowledgements

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Cysteine mutations in von Willebrand factor associated with increased clearance

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Abstract

Von Willebrand disease (VWD) is a bleeding disorder caused by the decrease of functional von Willebrand factor (VWF). Low levels of VWF can result from decreased synthesis, impaired secretion, increased clearance or combinations thereof. Several mutations lead to impaired synthesis or secretion of VWF, however, little is known about the survival of VWF in the circulation. To evaluate the effect of several VWF mutations on VWF clearance the effect of three cysteine mutations (C1130F, C1149R or C2671Y) on the in vivo survival of VWF was studied in patients carrying these mutations and in a VWF-deficient mice model.

In patients carrying these mutations we observed increased VWF-propeptide/mature VWF ratios and rapid disappearance of VWF from the circulation after desmopressin treatment. Detailed analysis of *in vivo* clearance of recombinant VWF in a VWF-deficient mice model revealed a four-fold increased clearance rate of the mutants. The mutations C1130F, C1149R and C2671Y are each associated with reduced survival of VWF in the circulation. Detailed analysis of recombinant mutant VWF demonstrated that increased clearance was not due to increased proteolysis by ADAMTS13. We did not identify functional or structural characteristics that the mutant proteins have in common and could be associated with the phenomenon of increased clearance. In conclusion, cysteine mutations in VWF may result in reduced *in vivo* survival. The observation that various mutations are associated with increased *in vivo* clearance may have major implications for the therapeutic strategies that rely on the rise of endogenous VWF after desmopressin administration.

Introduction

Von Willebrand factor (VWF) is a plasma glycoprotein that plays a dual role in haemostasis. Firstly, VWF forms a complex with coagulation factor VIII (FVIII), which is required for FVIII survival *in vivo*. Secondly, VWF contributes to platelet adhesion and aggregation by acting as a molecular bridge between subendothelial collagen and platelets (reviewed in (1)).

VWF is produced in megakaryocytes and endothelial cells, where it is subjected to extensive posttranslational modifications, including proteolytic separation of the precursor molecule in VWF-propeptide and mature VWF. VWF maturation involves C-terminal dimerization and VWF-propeptide dependent N-terminal multimerization via the formation of covalent intermolecular cystine bonds (1). The ability of VWF to support platelet adhesion and aggregation increases with multimer size (1). In the circulation, multimer size is controlled by several mechanisms, including proteolytic cleavage by ADAMTS13 (2,3). VWF is also subjected to *N*- and *O*-linked glycosylation and is one of the rare plasma proteins that carry the blood group antigens A, B and H (4). The VWF glycosylation profile is one of the determinants of VWF survival in the circulation (5,6). Besides altered glycosylation, amino acid substitutions in VWF may also affect clearance. Recently, we have used a mouse-model to demonstrate that the mutation R1205H leads to a strongly reduced survival of VWF in the circulation (7).

Defects in the VWF gene result in a bleeding disorder (von Willebrand disease, VWD) with variable penetrance. VWD can be categorized in qualitative VWF defects (type 2) and quantitative VWF deficiency (type 1 and type 3; partial and virtually complete deficiency, respectively) (1). Most mutations in the human mutation database that are associated with the quantitative deficiencies originate from null alleles (gene deletions, stop codons, frame shifts, and splice mutations), but missense mutations have also been reported. Some of these mutations lead to replacement of cysteine residues, like C1130F, C1149R and C2671Y (8,9). Mutations C1130F and C1149R are both located in the VWF D3 domain and are recognized as dominant negative mutations (9,10). Patients heterozygous for C1130F and C1149R display a pronounced quantitative VWF deficiency, a prolonged bleeding time and a history of moderate bleeding (9,11,12). Mutation C2671Y is located towards the carboxy-terminus of the molecule in the connective region between the C2 and

CK domains. The mutation has been reported for a single patient who is compound heterozygous for this mutation and a gene deletion. The patient displays a type 3 phenotype with VWF antigen (VWF:Ag) levels of 0.02-0.04 U/mL, and part of the residual circulating VWF protein consists of proteolyzed products (8,13).

Analysis of recombinant variants of C1130F and C1149R VWF revealed that these mutants lacked high molecular weight multimers, which defect could be corrected upon co-expression with recombinant wild-type VWF (rVWF-wt). In contrast, rVWF-C2671Y showed a normal multimerization pattern. All three mutations resulted in reduced secretion of VWF due to intracellular retention, and, at least for rVWF-C1149R, proteosomal degradation (9,10,12). However, the plasma VWF:Ag levels found in the patients were much lower than the VWF levels observed in the *in vitro* expression experiments (12). This indicates that the intracellular retention and degradation of the mutants only partly explain the low VWF levels in the patients. In the present study we explored the possibility that modified clearance also contributes to the reduced VWF levels in these patients. By investigating the behaviour of endogenous VWF upon desmopressin treatment of patients as well as the survival of recombinant VWF mutants in an experimental model employing VWF-deficient mice, we have indeed obtained evidence that increased clearance contributes to the reduced VWF levels *in vivo*.

Materials and methods

Patients, mutations and phenotypic tests

The C1130F and C1149R mutations were originally identified in patients classified as type 1 VWD, characterized by very low VWF:Ag levels (0.10–0.15 IU/mL) (9). The C2671Y mutation was found in a type 3 VWD patient, compound heterozygous for this mutation and a deletion of the other allele (8). Plasma was collected on several occasions from seven patients and two unaffected family members (Table I). An infusion with 1-deamino-8-D-arginine vasopressin (DDAVP; 0.3 µg DDAVP/kg body weight) was performed after informed consent. Blood samples were analyzed for VWF:Ag by an immunosorbent assay, VWF ristocetin cofactor (VWF:RCo) activity by aggregometry using fixed human platelets and FVIII coagulant (FVIII:C) activity using a one-stage clotting assay. The concentrations of

Table I: Patient characteristics

VWF	Family and patients*	FVIII:C (IU/mL)	VWF:Ag (IU/mL)	VWF:RCo (IU/mL)	Blood group
C1130F/wt	C proband	0.19	0.14	<0.20	O
	C sister	0.2-0.25	0.13-0.16	<0.20	O
	B proband	0.45	0.13-0.21	<0.20	A
C1149R/wt	III-4	0.18-0.21	0.10-0.21	<0.20	A
	IV-1	0.22-0.29	0.11-0.22	<0.20	A
	IV-2	0.35-0.74	0.15-0.35	<0.20	O
	III-5†	1.17	0.83-0.91	0.93	O
	IV-3†	1.09	0.86-1.33	0.91	A
C2671Y/del	A III-2	0.17	0.10	<0.20	O
Normal range		0.5-2	0.4-2	0.5-2	

*Patients and family members are indicated according to the original articles (8,9,11).
†Unaffected family members. FVIII:C, FVIII activity; VWF:Ag, VWF antigen; VWF:RCo, VWF ristocetin cofactor activity.

VWF-propeptide antigen were analyzed in an immunosorbent assay as described (14,15). A plasma pool containing 6.3 nM VWF-propeptide and 50 nM VWF (monomer concentration) was used as a standard.

Mice

The VWF-deficient (16) and wild-type mice were on a C57BL/6J background and were between 8 and 12 weeks old. Housing and experiments were done as recommended by French regulations and the experimental guidelines of the European Community

Proteins

Recombinant Glycoprotein Ibα (GpIbα, residues 1-290) was prepared as described (17). The GpIbα antibody (2D4) was a gift of H. Deckmyn (Kortrijk, Belgium). Botrocetin was from Kordia (Leiden, the Netherlands). Purified recombinant

VWF-propeptide was prepared as described (15). Recombinant B-domain deleted FVIII (Refacto) was from Wyeth. Human collagen type III (catalogue number C-4407) and bovine albumin (fraction V) were from Sigma. Human albumin (fraction V) was from MP Biochemicals (Irvine, CA, USA). Polyclonal antibodies (unlabeled and peroxidase-conjugated) against VWF were from Dako (Glostrup, Denmark).

Plasmids and recombinant expression of VWF

All plasmids were constructed using conventional techniques and all constructs were sequenced before transfection. Plasmids pSVHVWF encoding rVWF-wt, rVWF-C1130F, rVWF-C1149R and rVWF-C2671Y were transiently expressed in 293T human kidney cells (12). These recombinant variants were used to analyze ADAMTS13-mediated proteolysis. Plasmids pNUT-VWF encoding rVWF-wt, rVWF-C1130F, rVWF-C1149R and rVWF-C2671Y were stably expressed in baby hamster kidney cells overexpressing furin for proper removal of the VWF-propeptide (7,18,19). cDNA encoding VWF-wt was subcloned into pcDNA6 for co-transfection to rVWF-C1149R-expression cells, allowing selection using blasticidin to establish stable cell-lines expressing rVWF-wt and rVWF-C1149R heterozygously. All recombinant variants were purified from conditioned serum free medium, and used in the various functional assays and to determine VWF clearance in mice.

ADAMTS13-mediated proteolysis

The pcDNA3.1-ADAMTS13 V5/hisC expression vector (kindly provided by B.M. Luken and J. Voorberg, CLB research at Sanquin, Amsterdam, the Netherlands) was used for transient expression of recombinant ADAMTS13. Conditioned medium was collected and Pefabloc SC (Roche Diagnostics, Mannheim, Germany) was added to a final concentration of 1 mM. Medium was concentrated ~10 times by centrifugation in 5 mM Tris (pH 8.0) using Macrosep concentrators (cut-off value 100 kDa; Pall Gellman Laboratory, Ann Harbor, USA). ADAMTS13 protease activity was determined using previously described methods and conditions (20,21). The sensitivity of a fixed concentration of mutant, co-transfected or wt VWF was assessed with a 1:20 dilution of ADAMTS13 in 5 mM Tris (pH 8.0) with

12.5 mM $BaCl_2$ to initiate the reaction. The final concentration of urea in the reaction was 0, 0.1, 0.5, 1.0 or 1.5 M. Aliquots were taken after 0, 8 and 24 h incubation at 37°C and the reaction was stopped with EDTA. Subsequently, the VWF multimeric structure was analyzed on denaturing, non-reducing agarose gels (1.5% (w/v)) (12,22). In all tests the same batch of concentrated ADAMTS13 was used.

Clearance of purified recombinant VWF in mice

Clearance of recombinant wt and mutant VWF has been analyzed as described (7,16). Three to six mice were used for each time-point, and each mouse was bled only once. Human VWF:Ag levels were quantified as described (14).

GpIbα binding

Monoclonal anti-GpIbα antibody 2D4 was immobilized in microtiter-wells (Costar, Cambridge MA, USA) in 50 mM $NaHCO_3$ (1.0 µg/mL, overnight at 4°C), which were then blocked for 1h at 37°C with PBS/3% (w/v) bovine albumin/0.1% (v/v) Tween-20). Recombinant GpIbα was added (0.1 µg/mL for 2 h at 37°C) and subsequently increasing concentrations of VWF (0-4 nM) were applied in the presence of botrocetin (2 µg/mL) and incubated for 2 h at 37°C. After washing, wells were incubated with peroxidase-labeled polyclonal anti-VWF antibodies (1.3 µg/mL for 1 h at 37°C), and bound VWF was detected by measuring peroxidase activity using *O*-phenylenediamine as a substrate.

αIIB β3-dependent platelet adhesion to immobilized VWF

Perfusion with platelets (shear rate 1600 s^{-1}) over VWF-coated cover slips was performed as described elsewhere (23). The amount of platelet-adhesion was evaluated using computer-assisted analysis with OPTIMAS-6.0 software (Dutch-Vision-Systems, Breda, the Netherlands), and was expressed as the percentage of surface-coverage. Perfusions were performed 6-9 times for all variants.

Surface Plasmon Resonance analysis

Several binding assays were performed employing a Biacore2000 system (Biacore AB, Uppsala, Sweden). Binding of VWF-propeptide and FVIII to immobilized VWF or its mutants was investigated as described (7), except that recombinant B-domain deleted FVIII (Refacto) was used instead of FVIII light chain. Binding of VWF or its derivatives to immobilized collagen type III was performed as described (14).

Data analysis and statistics

Analysis of protein-interaction assays and clearance data obtained from mouse experiments was performed using the GraphPad Prism program (GraphPad Prism version 4.0 for Windows, GraphPad Software, San Diego, CA, USA) as described (7). Clearance data obtained from the patients, after subtraction of the basal VWF and VWF-propeptide level from the post-DDAVP values, were fitted to the monoexponential equation $C_t = Ae^{-\alpha t}$ to obtain α. C_t refers to the plasma concentration of VWF or VWF-propeptide at time-point t after DDAVP infusion, and the apparent half-life was calculated from the equation $t_{½} = \ln 2/\alpha$. Statistical analyses were performed by the Student's unpaired t-test using the GraphPad InStat program (GraphPad InStat version 3.00 for Windows).

Results

Increased VWF-propeptide/mature VWF ratio

VWF is secreted simultaneously with its propeptide at equimolar concentrations and they circulate at a distinct ratio (15,24). The patients in this study display VWF:Ag levels that are well below the normal range (Table I). In contrast, the VWF-propeptide levels were less reduced, resulting in an increased ratio of VWF-propeptide over VWF:Ag (Fig. 1). These ratios were increased three-fold for patients with the mutations C1130F and C1149R compared to the normal population. The ratio was also increased in the compound heterozygous patient (C2671Y/deletion) (Fig. 1). These data suggest a reduced half-life of VWF, or, alternatively, a prolonged half-life of the VWF-propeptide.

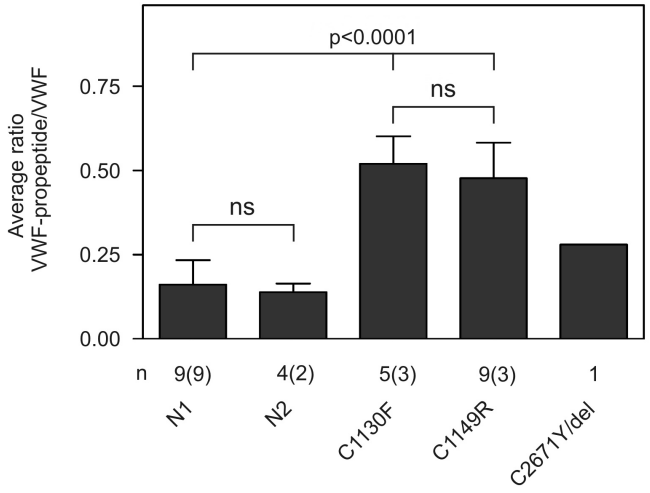

Fig. 1, VWF-propeptide/VWF ratio in VWD patients. The average ratio of VWF-propeptide over mature VWF for normal controls (N1), unaffected family members from the family with the C1149R mutation (N2), and patients harbouring the C1130F, C1149R or C2671Y/del mutation at basal level are depicted. The number of measurements (n) is indicated, with the number of patients in brackets. Differences between groups were tested with the unpaired two-tailed t-test. Data represent mean ± standard deviation, and data of N1 were obtained in a previous study (24). ns, not significant (p>0.05).

Increased clearance of VWF upon DDAVP treatment

To test whether the modified ratios result from a change in half-life of VWF-propeptide or mature VWF, plasma levels of VWF and VWF-propeptide were monitored at different time-points after DDAVP infusion. Healthy subjects and patients showed a rapid increase of the VWF-propeptide level, which was followed by a monophasic decay (Fig. 2A). The half-life of VWF-propeptide in patients with C1130F or C1149R was similar to healthy subjects, whereas the half-life of VWF-propeptide in the patient with C2671Y was somewhat longer (Fig. 2A, Table II). VWF showed a similar rapid increase followed by a monophasic decay (Fig. 2B). In the patient group, VWF was cleared much quicker than in the group of healthy subjects. Indeed, the estimated half-life of VWF in the patients with mutations C1130F, C1149R and C2671Y was reduced four to five-fold (Fig. 2B, Table II). It should be noted that the estimated half-lives were based on a limited number of time points, which prevents accurate calculation of the half-lives.

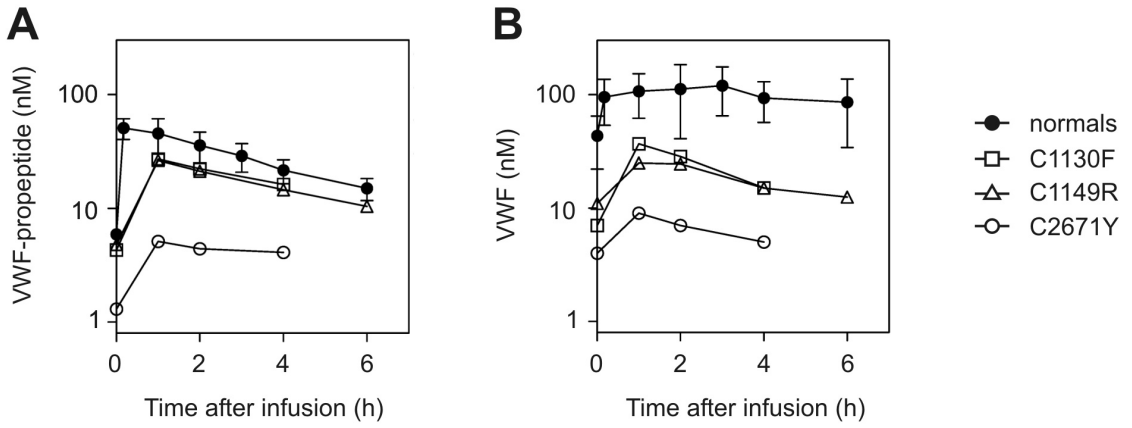

Fig. 2, survival of endogenous VWF-propeptide and VWF after DDAVP administration. Blood was collected before and after DDAVP infusion (0.3 µg/kg body weight). Samples were analyzed for VWF-propeptide (**A**) and VWF (**B**) levels. Shown are the mean ± standard deviation for controls (n=9) and data from single experiments in patients. Data of the normal controls were obtained in a previous study (24). The apparent half-lives are listed in Table II.

Table II: Apparent half-lives of VWF-propeptide and VWF after DDAVP treatment

VWF	$t_{½}$ VWF-propeptide (h)	$t_{½}$ VWF (h)	Blood group
Normal	2.3	7.0	
C1130F	3.2	1.6	O
C1149R	2.6	1.5	A
C2671Y	6.0	1.3	O

Nevertheless, these data strongly indicate that the mutations C1130F, C1149R and C2671Y are associated with an increased clearance of mature VWF.

Mutations at C1130, C1149 and C2671 leave ADAMTS13-dependent proteolysis unaffected

Rapid clearance could be due to increased susceptibility of VWF mutants to degradation by ADAMTS13. The susceptibility of the recombinant mutants for ADAMTS13 was therefore assessed in degradation assays. Representative results from the protease assay of rVWF-C1130F and rVWF-C2671Y are shown in Fig. 3. No or little degradation was detected in the absence of urea. However, the presence

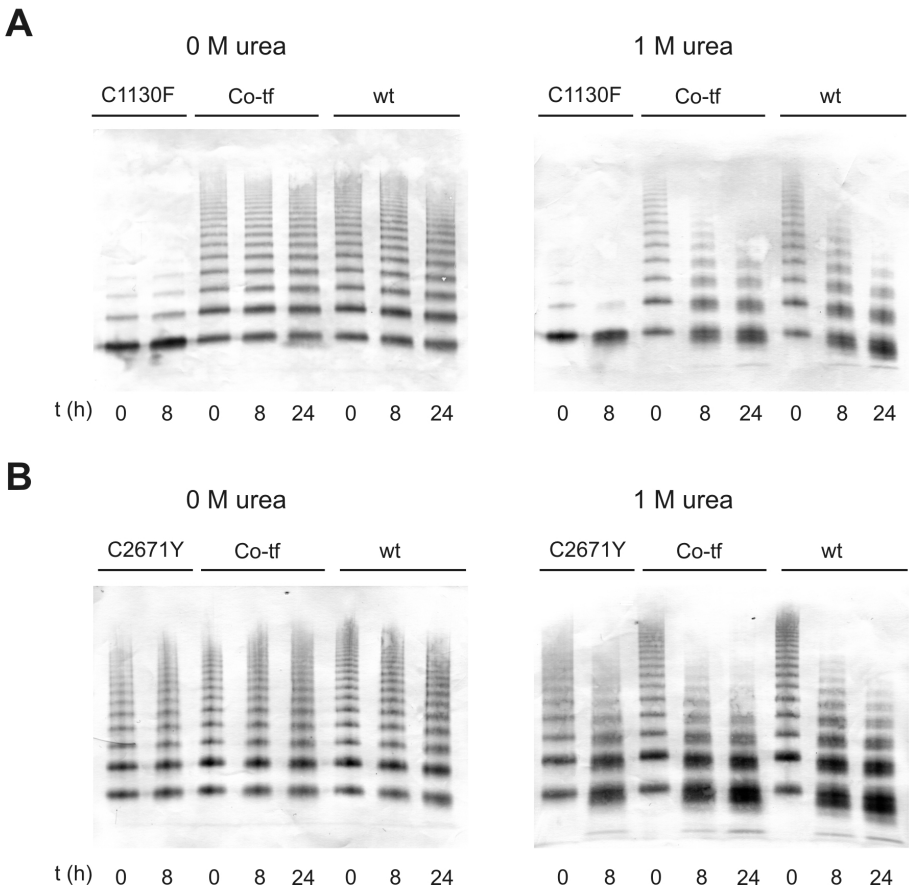

Fig. 3, ADAMTS13-mediated proteolysis of recombinant wild-type and mutant VWF. rVWF-C1130F (**A**), rVWF-C2671Y (**B**), rVWF-wt (wt) alone or VWF obtained from co-transfections (Co-tf) were incubated with recombinant ADAMTS13 for 0, 8 or 24 h (t) in the absence or presence of 0.1, 0.5, 1.0, 1.5 M urea. The results from 0 and 1.0 M urea are depicted. The degree of proteolysis was assayed on SDS-agarose gel electrophoresis under non-reducing conditions. The results for rVWF-C1130F are representative for rVWF-C1149R (data not shown).

of 1.0 M urea resulted in a gradual disappearance of the higher multimers (Fig. 3). Due to impaired multimerization of rVWF-C1130F and rVWF-C1149R, the sensitivity for ADAMTS13 could only be interpreted for the respective co-transfections with wt VWF, which are also more representative for the situation in the heterozygous patients (Fig. 3). At all urea concentrations and at all incubation times tested, the VWF mutants were indistinguishable from wt VWF and no evidence was found that the accelerated disappearance from the

circulation is caused by an elevated susceptibility towards ADAMTS13-mediated cleavage.

Increased clearance of VWF mutants in VWF-deficient mice

The clearance of mutant VWF was studied in a murine model in which VWF clearance is independent of ADAMTS13-mediated proteolysis and independent of the extent of VWF multimerization (7). VWF mutants produced by stable cell-lines were purified from conditioned medium. Multimer analysis revealed a normal multimeric pattern for rVWF-C2671Y. rVWF-C1130F and rVWF-C1149R consisted mainly of low molecular weight multimers, whereas co-expression of rVWF-C1149R with rVWF-wt resulted in the reappearance of higher multimers. These patterns are similar to that of the mutants produced by transiently transfected cells (Fig. 3 and reference (12)). Purified proteins were injected intravenously in VWF-deficient mice and residual plasma levels were measured. The mutants and wt VWF displayed a similar recovery after injection (Table III). In contrast, all four mutants were cleared from the circulation more rapidly than wt VWF (Table III, Fig. 4). In the murine system, wt VWF disappears from plasma in a biphasic manner, characterized by a rapid initial phase and a slow secondary

Table III: Pharmacokinetic parameters of the clearance of recombinant VWF in mice

rVWF	Recovery (% of injected)	Mean residence time (h)	$t_{½}\alpha$ (min)	$t_{½}\beta$ (h)
wt*	79 ± 14	2.8 ± 0.7	12.6 ± 0.9	3.0 ± 0.9
D2509G*	82 ± 5 (p>0.05)	2.3 ± 0.5 (p>0.05)	12.0 ± 2.2 (p>0.05)	2.2 ± 0.2 (p>0.05)
C1130F	72 ± 6 (p>0.05)	0.7 ± 0.2 (p=0.007)	6.0 ± 1.4 (p=0.002)	1.1 ± 0.5 (p=0.032)
C1149R	67 ± 10 (p>0.05)	0.8 ± 0.4 (p=0.011)	7.5 ± 2.5 (p=0.028)	1.1 ± 0.5 (p=0.032)
wt/C1149R	75 ± 9 (p>0.05)	0.4 ± 0.1 (p=0.0042)	5.5 ± 0.4 (p=0.0002)	0.7 ± 0.1 (p=0.012)
R1205H*	97 ± 8 (p>0.05)	0.3 ± 0.1 (p=0.004)	7.6 ± 0.2 (p=0.0007)	0.3 ± 0.03 (p=0.007)
C2671Y	63 ± 8 (p>0.05)	0.7 ± 0.1 (p=0.006)	8.9 ± 1.5 (p=0.022)	0.7 ± 0.2 (p=0.012)

*Data were obtained during a previous study (7). rVWF-wt is reference for comparisons. rVWF-D2509G and rVWF-R1205H are shown to illustrate the normal and decreased half-life of other recombinant mutants.

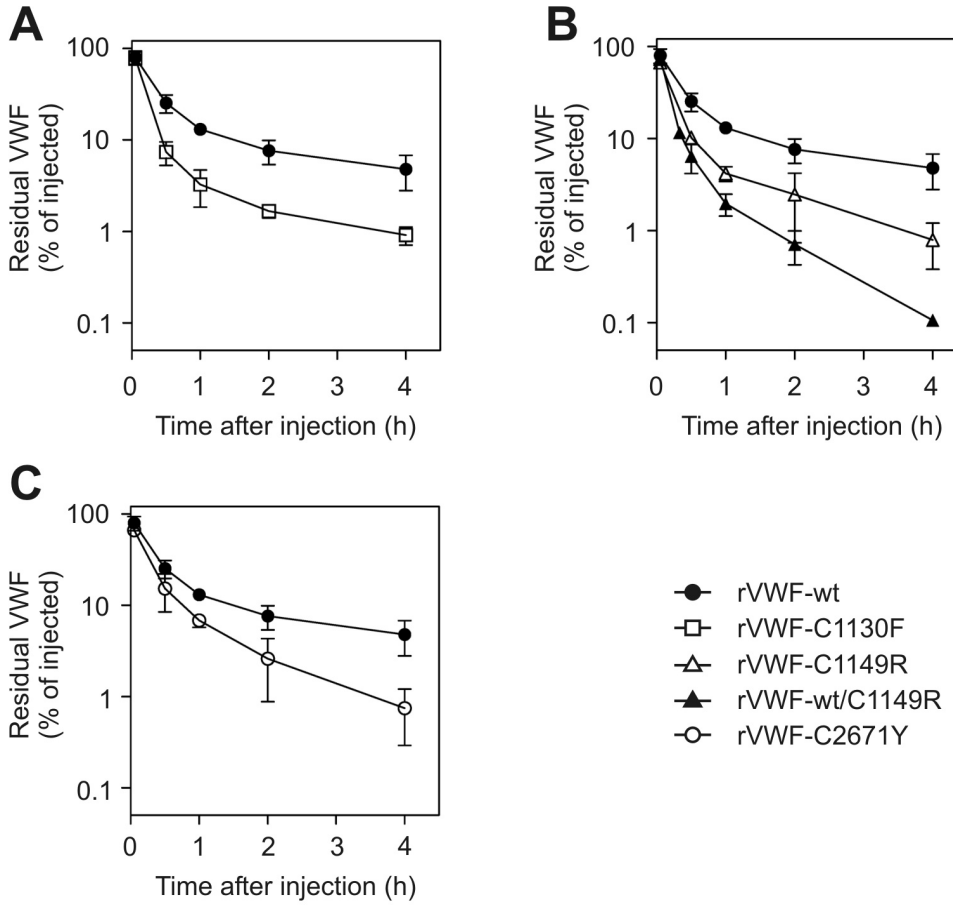

Fig. 4, *in vivo* survival of recombinant VWF mutants. VWF-deficient mice were injected intravenously with purified rVWF-wt, rVWF-C1130F (**A**), rVWF-C1149R (**B**), rVWF-wt/C1149R (**B**) or rVWF-C2671Y (**C**) at a concentration of 5 µg/mouse. Plotted is the residual VWF:Ag in plasma relative to the amount injected *versus* time after injection. For clarity, only data between 3 min and 4 h are shown. Data for rVWF-wt were obtained in a previous study, and are shown for comparison. Pharmacokinetic parameters derived from the complete data set (from 3 min to 24 h) are summarized in Table III. Data represent the mean ± standard deviation of 3-6 mice for each time-point.

phase (7). As for wt VWF, the mutants disappeared in a biphasic manner, but data analysis revealed that the initial rapid phase ($t_{½}\alpha$), the slow secondary phase ($t_{½}\beta$) and mean residence time were significantly reduced compared with the values obtained for wt VWF (Table III). Thus, the mutations C1130F, C1149R and C2671Y *per se* are associated with accelerated clearance of VWF.

Functional characterization of rVWF-C1130F, rVWF-C1149R and rVWF-C2671Y

Because the accelerated clearance of the mutant VWF is due to the mutations *per se*, we examined whether the mutants have functional or structural characteristics in common that point to a region in VWF responsible for the increased clearance. The results of a number of functional parameters (*i.e.*, FVIII-, GpIbα- and collagen-binding, platelet-adhesion under flow-conditions) are summarized in Table IV. Briefly, mutant rVWF-C2671Y was similar to rVWF-wt in all assays tested. Mutant rVWF-C1130F displayed normal GpIbα-binding and platelet-adhesion capacity. Furthermore, FVIII-binding was reduced five-fold for this mutant, while collagen-binding was reduced to a level similar to a dimeric control protein. Mutant rVWF-C1149R was most severely affected; apart from collagen-binding that was reduced to the level of the dimeric control, all other functions were considerably reduced compared to the control proteins.

In view of the multimerization defects of rVWF-C1130F and rVWF-C1149R, we also tested the interaction with the VWF-propeptide. This interaction mediates intracellular multimerization and targeting of mature VWF to the storage organelles and occurs under slightly acidic conditions. The interaction between VWF and VWF-propeptide was investigated at various pHs (Fig. 5). For rVWF-wt a pH-dependent increase in affinity was observed with lower affinity at higher pH (pH 7.4, $K_{D,\,app}$ = 1.0 ± 0.1 μM and pH 5.2, $K_{D,\,app}$ = 0.08 ± 0.02 μM). A similar pattern was found for rVWF-C2671Y. However, binding of VWF-propeptide was

Table IV: Functional analysis of recombinant VWF mutants

rVWF	GpIbα binding Half-maximal binding (nM)	Collagen binding $K_{D,\,app}$ (nM)	FVIII binding $K_{D,\,app}$ (nM)	Platelet adhesion Surface coverage (%)
wt	1.2 ± 0.2	9.4 ± 0.6	0.51 ± 0.04	77.3 ± 6.3
C1130F	1.0 ± 0.1	47.4 ± 1.0	2.3 ± 0.1	80.9 ± 8.8
C1149R	3.9 ± 1.1	53.4 ± 1.4	10.6 ± 0.4	49.4 ± 16.1
C2671Y	1.0 ± 0.2	8.3 ± 0.6	0.33 ± 0.02	79.2 ± 9.8
Dimeric control*	nd	67.5 ± 1.2	0.38 ± 0.06	70.5 ± 6.4

*Dimeric control was: rVWF-(D'-A3) for collagen binding, rVWF-(A1-CK) for platelet-adhesion and rVWF-(D'-D3) for FVIII binding. All dimeric controls have been described previously (7). nd, not determined.

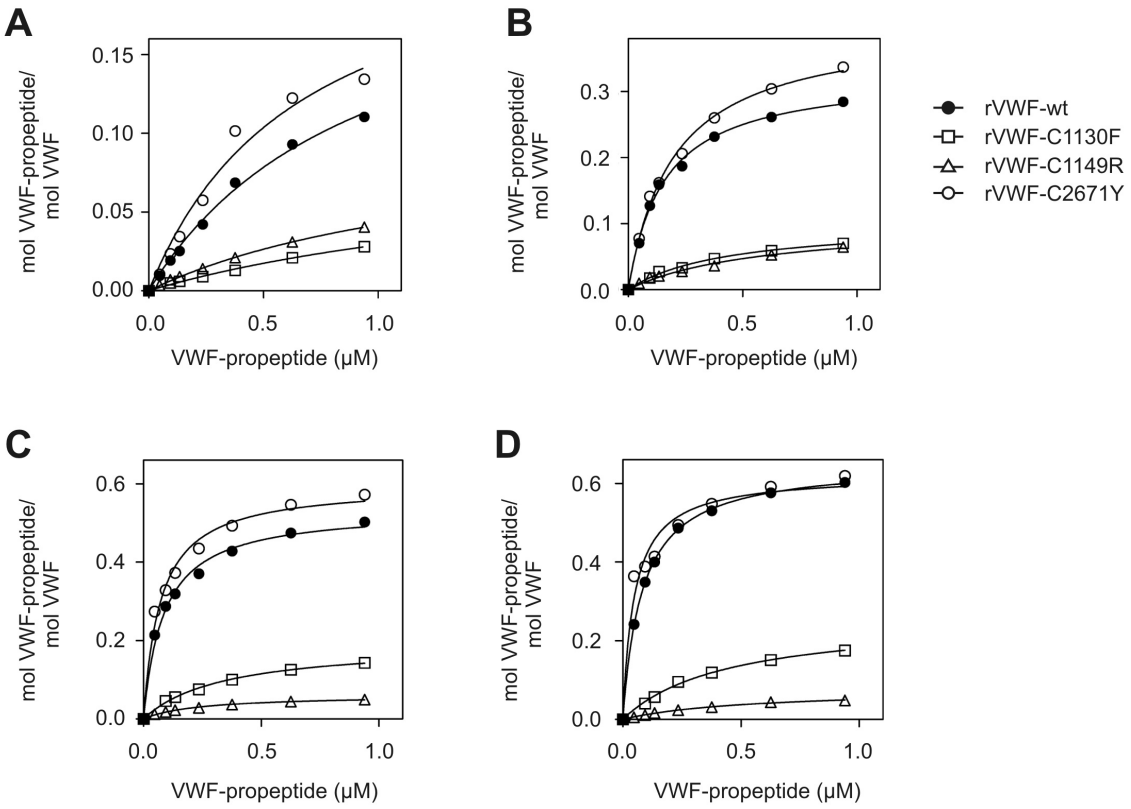

Fig. 5, binding of VWF-propeptide to immobilized VWF. rVWF-wt, rVWF-C1130F, rVWF-C1149R, and rVWF-C2671Y immobilized onto a CM5 sensor-chip (10-12 fmol/mm^2) were incubated with various concentrations of purified VWF-propeptide (0-1.0 μM). Incubations were performed in 125 mM NaCl, 25 mM HEPES (pH 7.4 (**A**) or pH 6.8 (**B**)), 125 mM NaCl, 25 mM $(CH_3)_2AsO_2Na$ (pH 5.8 (**C**) or pH 5.2 (**D**)) at a flow rate of 10 μl/min for 4 min at 25°C. Response at equilibrium (indicated as mol VWF-propeptide/mol VWF) is plotted against concentration of VWF-propeptide. Note the different scales of the Y-axes. Data represent the mean of duplicate experiments (range less than 10 %).

markedly reduced for mutants rVWF-C1130F and rVWF-C1149R ($K_{D,\ app}$ > 2 μM at all pHs tested). Again, rVWF-C1149R was less efficient compared with rVWF-C1130F. These data indicate that mutations within the D'-D3 domains are not only associated with increased clearance, but also may lead to suboptimal interactions with ligands that bind to this particular region of the VWF molecule.

Discussion

Circulating levels of VWF need strict regulation, as levels that are too low are associated with an increased bleeding tendency (1), whereas levels that are too high predispose to an increased risk of cardiovascular mortality (25). Low levels of VWF can result from decreased synthesis, impaired secretion, increased clearance or combinations thereof. Several mutations lead to impaired synthesis or secretion of VWF, however, little is known about the relationship between amino acid variations in VWF and its survival in the circulation. In the present study we evaluated the effect of three VWD-associated mutations on the survival of VWF. These mutations were selected on basis of discrepancies between VWF levels measured in *in vitro* expression experiments and the relatively lower actual antigen values in the patients' plasmas (9,10,12). Such discrepancies may indicate the presence of mechanisms other than synthesis and secretion that contribute to the low VWF levels.

We have obtained several lines of evidence of increased clearance of mutant C1130F, C1149R and C2671Y VWF. Firstly we analyzed in the patients the VWF-propeptide/VWF:Ag ratio, which is the resultant of a dissimilar survival of these proteins in the circulation (15,24). The VWF:Ag levels were much more reduced than the VWF-propeptide levels, resulting in ratios that were increased up to three-fold compared with normal individuals or unaffected family members (Fig. 1). Secondly, VWF disappeared from the circulation four to five-fold more rapidly in the patients upon DDAVP treatment (Fig. 2B). Thirdly, we performed detailed analysis of *in vivo* clearance of recombinant VWF in a model employing VWF-deficient mice (7). Both the initial and secondary phase of the clearance of rVWF-C1130F, rVWF-C1149R and rVWF-C2671Y were accelerated (Fig. 4), which was reflected by a mean residence time that was reduced four-fold (Table III). Thus, the mutations C1130F, C1149R and C2671Y are each associated with a reduced survival of VWF in the circulation. Because also the heterozygous rVWF-wt/C1149R mimicked the increased clearance of the protein in the patients, it seems that at least some of these mutations may have a predominant effect on the clearance of the mutated protein. Furthermore, we have recently shown that also mutation R1205H results in increased clearance of VWF (7). A preliminary report has recently described a mutation S2179F in the VWF D4 domain, which seems to have a similar effect on VWF clearance (26). This indicates that several

mutations in VWF predispose to increased clearance. Indeed, a decreased survival of VWF upon DDAVP treatment in a cohort of VWD type 1 patients has recently been reported (27).

This conclusion is of importance with respect to treatment of VWD patients. The goal of treatment is to correct the deficiency of VWF and FVIII, either by transfusion with plasma-derived FVIII/VWF concentrates or DDAVP administration. The latter leads to the release of endogenous VWF from storage organelles, which are associated with a three to five-fold increase of VWF levels. However, if due to a mutation the survival of endogenous VWF is reduced, the initial response to DDAVP may be normal, but the effect is only short-lasting because the endogenous VWF is cleared rapidly. In some of these patients it may be more appropriate to treat with FVIII/VWF concentrates. As also put forward by Brown *et al.* (27), the clinical practice to judge the effectiveness of DDAVP treatment by the initial rise of VWF may be insufficient for several patients as it does not take clearance into account. Therefore we suggest that, after a DDAVP test-infusion, VWF:Ag is monitored over a sufficient length of time and we propose the implementation of the VWF-propeptide/VWF:Ag ratio in the diagnosis of VWD.

The question remains why the various mutations are associated with increased clearance of the VWF protein. It has recently been shown that the presence of a Tyr to Cys polymorphism at position 1584 results in increased susceptibility to the VWF-cleaving protease ADAMTS13 (28). As the patient harbouring the C2671Y mutation displays relatively high plasma levels of VWF degradation products (13), we tested whether increased clearance could result from an increased susceptibility to the ADAMTS13 protease. However, we did not find evidence of increased proteolysis (Fig. 3). Moreover, normal proteolysis was also reported for VWF/R1205H (29), another mutant that displays increased clearance (7). Final evidence that the increased clearance of the various mutants is independent of ADAMTS13 was provided by the rapid clearance in the mouse model, as murine ADAMTS13 does not recognize human VWF. It should be noted that our study has not examined the potential role of thrombospondin-1, a plasma depolymerase that is able to modulate VWF multimer size (30).

Increased clearance may also be due to disturbed structural integrity of the mutant proteins, rendering them susceptible to quality control mechanisms that remove anomalous proteins from the circulation. We have tried to identify

whether the mutant proteins have functional or structural characteristics in common that may be associated with the phenomenon of increased clearance. Several parameters were examined, but none of these functional tests pointed to a particular region within the VWF molecule that seems to be consistently associated with abnormal clearance. Mutant rVWF-C2671Y was normal for all functions, indicating that this mutation results in local changes within the molecule only. This could suggest that this part of VWF contributes to interactions with clearance receptors. All other mutations that we tested so far, *i.e.*, rVWF-C1130F, rVWF-C1149R and rVWF-R1205H, are located within a relatively short stretch in the D3 domain. However, the impact of these mutations on VWF function differs considerably. Whereas mutant rVWF-R1205H has normal interaction with FVIII (7), both rVWF-C1130F and rVWF-C1149R display impaired FVIII binding (Table IV). Mutations C1130F and C1149R further resulted in a lack of proper multimerization (Fig. 3, reference (12)), which was not seen with rVWF-R1205H (7). A combined effect on FVIII binding and multimerization has been described for several other mutations, and seems to be related to a local distortion of the secondary structure (31). Incomplete multimerization is a consequence of a suboptimal interaction with the VWF-propeptide, which is involved in intracellular multimerization of VWF (32,33). Indeed, mutants rVWF-C1130F and rVWF-C1149R displayed defective binding of the VWF-propeptide (Fig. 5). Thus, the lack of multimerization observed for these mutants can readily be explained by an almost complete absence of VWF-propeptide binding.

In conclusion, our study shows that certain amino acid changes within the VWF molecule are associated with increased clearance. This may have major implications for the therapeutic strategies that rely on the temporary rise of endogenous VWF after DDAVP administration. New therapeutic approaches based on the inhibition of VWF clearance could be developed as an adjuvant to optimize treatment of these patients. However, more insights into the molecular mechanisms mediating VWF clearance are needed in this regard.

Addendum

P.J. Lenting, and C.V. Denis: conception and design of the study, performing experiments, analysis of results, drafting and final version of the manuscript. J.C.J. Eikenboom: conception and design of the study, analysis of results, drafting and final version of the manuscript. C.J. van Schooten, P. Tjernberg, E. Westein, V. Terraube and M.J. Hollestelle: design of the study, performing experiments, analysis of results, drafting and final version of the manuscript. G. Castaman, and J.A. van Mourik: data collection of patients and normal controls respectively, responsible for DDAVP infusion experiments, revision of the draft and approval of final manuscript. H.L. Vos, R.M. Bertina, and H.M. van den Berg: design of the study, revision of the draft and approval of final manuscript.

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Homozygous C2362F von Willebrand factor induces intracellular retention of mutant von Willebrand factor resulting in autosomal recessive severe von Willebrand disease

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Abstract

The missense mutation of cysteine 2362 to a phenylalanine in von Willebrand factor (VWF) has been detected in several Italian families with autosomal recessive, severe von Willebrand disease. We investigated how this amino acid change in VWF may lead to a predominantly quantitative defect. This mutation was studied *in vitro* by transient expression of the full-length mutant VWF-C2362F protein and *in vivo* by analysis of plasma VWF after infusion of 1-deamino-8-D-arginine vasopressin in a patient homozygous for this mutation. Single transfections of pSVHVWF-C2362F and co-transfections of mutant and wild-type constructs resulted in 8% and 50% VWF antigen, respectively, in conditioned medium. These reduced levels are in accordance with observations in homozygous and heterozygous carriers of the mutation. In addition, VWF-C2362F was retained intracellularly. Similar results were obtained for VWF-C2362A. After infusion of 1-deamino-8-D-arginine vasopressin in a patient homozygous for the C2362F mutation, a two-fold decrease in half-life of plasma VWF-C2362F was observed. This was not explained by increased susceptibility of recombinant VWF-C2362F to ADAMTS13. It was concluded that VWF-C2362F causes reduced VWF plasma levels due to impaired secretion and intracellular retention. Furthermore, it is the loss of cysteine 2362 rather than the introduction of the bulky amino acid side chain that causes these effects.

Introduction

Von Willebrand factor (VWF) is a high molecular weight (HMW) multimeric glycoprotein (0.5-10x10^6 Da) with adhesive properties. In the circulation, VWF mediates adhesion of platelets to the vessel wall and platelet-platelet aggregation at the site of injury. VWF also functions as a carrier of factor VIII (FVIII). VWF and FVIII form a non-covalent complex, which prolongs the half-life of FVIII in the circulation reviewed in (1,2).

Von Willebrand disease (VWD) is the most common inherited bleeding disorder. It is caused by dysfunction or deficiency of VWF. VWD is divided into qualitative (type 2) and quantitative (types 1 and 3) VWF defects (3). The molecular basis of most of the qualitative VWD variants have been identified (4). However, the identification of mutations causing quantitative VWD has been more difficult due to the occurrence of such mutations throughout the whole VWF gene.

Type 3 VWD is characterized by virtually complete deficiency of VWF, autosomal recessive inheritance, and moderate to severe bleeding symptoms. Heterozygous carriers of type 3 mutations have about 50% VWF antigen (VWF:Ag) and usually only mild or no bleeding symptoms. In addition to the many mutations that result in a null allele, a few missense mutations have also been identified in type 3 VWD (5). Compound heterozygosity and homozygosity for missense mutations of cysteine residues 2739, 2754, 2804 and 2806 have been described in type 3 VWD (6-9). All these mutations are located in the carboxy-terminal cystine knot (CK) domain. Another mutation of a cysteine residue that causes a major quantitative deficiency is the C2362F mutation, which is found in several Italian patients with autosomal recessive severe VWD (10). Cysteine 2362 is particularly interesting as it is located in a region of VWF of which the function is yet unknown. The location of C2362 outside the amino-terminal region and the CK domain, involved in the multimerization and dimerization of VWF, respectively, excludes its participation in an interchain disulfide bond.

The present study investigated the effect of the C2362F mutation on the quantity and quality of the VWF produced. It was hypothesized that the quantitative defect observed in patients homozygous for the C2362F mutation is the result of intracellular retention and rapid degradation of a VWF subunit which

is misfolded due to the loss of an intrachain disulfide bond. To evaluate whether the replacement of a cysteine by the more bulky and hydrophobic phenylalanine residue may additionally compromise the conformation of the mutant subunit, both C2362F and the C2362A, were studied. In addition, we assessed aspects of clearance of C2362F by the *in vivo* survival of plasma VWF-C2362F after infusion of 1-deamino-8-D-arginine vasopressin (DDAVP) and by studying the *in vitro* susceptibility of recombinant VWF (rVWF)-C2362F to proteolysis by the VWF-cleaving protease ADAMTS13.

Materials and methods

Patients and mutations

The C2362F mutation was previously found in autosomal recessive severe VWD patients, characterized by very low levels of VWF:Ag (0.02-0.07 IU/mL) and a moderate to severe bleeding tendency (10,11). Two patients were studied. One patient was homozygous for the C2362F mutation (patient M III-2 in reference (10)). The other patient was compound heterozygous for the C2362F mutation in combination with a C to A substitution at position 11/24 in the consensus sequence of the 3' splice site of intron 13, which leads to skipping of exon 14 and a premature stop codon (patient III-2 in reference (12)). A third subject was heterozygous for the C2362F mutation and had mildly decreased FVIII activity (FVIII:C), VWF:Ag and VWF ristocetin cofactor (VWF:RCo) activity, but a normal bleeding time (subject II-5 in reference (12)). All three patients were identified in the Veneto region in northern Italy and the allele carrying the C2362F mutation is identical for five additional polymorphic markers in all patients suggesting the presence of a founder allele (10,12). The patients' characteristics are presented in Table I.

DDAVP infusion and VWF parameters

The patient homozygous for the C2362F mutation was infused with DDAVP (dosage 0.3 µg DDAVP/kg body weight) after informed consent. Blood was drawn before and 1, 2 and 4 hours after starting the DDAVP infusion. The patient's plasma was analyzed for VWF:Ag by a standard enzyme-linked immunosorbent

Table I: Patient characteristics

			PFA† (s)								Platelet (U/10^{11} platelets)			
Genotype of subject	DDAVP (h)	BT* (min)	ADP	EPI	FVIII:C (IU/mL)	VWF:Ag (IU/mL)	VWF:RCo (U/mL)	VWF:CB/ VWF:Ag§	VWF-propeptide (nM)	Ratio VWF-propeptide/ VWF (nM/nM)	VWF:Ag	VWF:RCo	Blood group	Bleeding tendency, bleeding score**
Homozygous††	0	>20	>300	>300	0.35	0.12	<0.03	0.76	0.43	0.07	<3	<3	A	Moderate-severe, 18
	1	>20	>300	>300	0.52	0.18	0.07	0.62	2.57	0.29	nd	nd		na
	2	nd	>300	>300	0.58	0.17	0.07	0.88	2.22	0.26	nd	nd		na
	4	>20	>300	>300	0.55	0.15	0.05	0.88	1.54	0.20	nd	nd		na
Compound heterozygous§§	-	>15	>300	>300	0.24	0.08	<0.03	0.64	0.64	0.16	3.5	3	B	Moderate, 6
Heterozygous††	-	4	102	117	0.41	0.37	0.31	1.07	3.99	0.21	21	18	O	None, -1
Normal range***		<7.5	<165	<165	0.52-1.73	0.47-1.65	0.51-1.88	0.80-1.13	3.2-7.8	0.015-0.307	17-88	24-102	-	<3

nd, not determined; na, not applicable; FVIII:C, factor VIIII coagulant activity; VWF:Ag, von Willebrand factor antigen; VWF:RCo, von Willebrand factor ristocetin co-factor. *BT, bleeding time (Simplate II; General Diagnostics, Morris Plains, NJ, USA). †PFA, platelet function analyzer (PFA-100; Dade Behring, Liederbach, Germany). §VWF:CB, VWF collagen binding activity/VWF:Ag ratio were determined according to (13). **Bleeding scores were calculated according to (14). ††Female. §§Male. ***Normal range of FVIII:C, VWF:Ag, VWF:RCo are from (12), platelet VWF:Ag and platelet VWF:RCo are from (15), the VWF:CB/VWF:Ag ratio are from (13) and VWF-propeptide and the basal VWF-propeptide/VWF ratio was reported previously (16,17).

assay (ELISA) method, for VWF:RCo activity by aggregometry using formalin-fixed human platelets and for FVIII coagulant activity (FVIII:C) by a one-stage clotting assay on an automated coagulometer. The concentration of VWF-propeptide was analyzed in an immunosorbent assay as described previously (18). A pooled normal plasma containing 6.3 nM VWF-propeptide and 50 nM VWF (based on concentration of VWF monomers) was used as a standard. After subtraction of the basal VWF and VWF-propeptide levels from the post-DDAVP values, the data were fitted to the monoexponential equation $C_t = Ae^{-\alpha t}$ to obtain α. C_t refers to the plasma concentration of VWF or VWF-propeptide at time-point t after DDAVP infusion, and the apparent half-life was calculated from the equation $t_{½} = \ln 2/\alpha$.

Plasmid construction

Construction of plasmid pSVHVWF encoding rVWF-wild type (wt) was described before (19). The C2362F and C2362A VWF mutations were introduced in the pSVHVWF plasmid containing the full-length cDNA of wt VWF. First, the wt *Hind*III-*Mun*I fragment of pSVHVWF was subcloned in pSE280 (Invitrogen, Groningen, the Netherlands), creating pSE280-*Hind*III-*Mun*I-wt. Then oligonucleotides 1 and 2 or oligonucleotides 3 and 4 (Table II) and pSE280-*Hind*III-*Mun*I-wt were used in combination with the QuikChange® XL site-directed mutagenesis kit from Stratagene (La Jolla, CA, USA) to introduce the C2362F or C2362A mutation using the following cycling conditions: denaturation at 95°C for 1 minute, and 18 cycles of denaturation at 95°C for 50 seconds, annealing at 60°C

Table II. Oligonucleotides for mutagenesis and sequencing

Oligonucleotide #	Direction (5' to 3')	Sequence*	Complementarity (to pSVHVWF)†	Purpose§
1	Forward	CCAACTTCACCTGCGCaTtCAGGAAGGAGGAGTGC	7337-7371	Mut
2	Reverse	GCACTCCTCCTTCCTGaAtGCGCAGGTGAAGTTGG	7371-7337	Mut
3	Forward	CCAACTTCACCTGCGCCgcCAGGAAGGAGGAGTGC	7337-7371	Mut
4	Reverse	GCACTCCTCCTTCCTGgcGGCGCAGGTGAAGTTGG	7371-7337	Mut
5	Forward	GCATTGGTGAGGATGGAGTCC	7040-7060	Seq
6	Reverse	GCAGATGGCAGGCACCTTCC	7763-7744	Seq

*Nucleotide substitution indicated in lower case, the additional *Nsb*I (tgcgca) or *Tau*I (gc c/g gc) restriction site introduced in pSVHVWF-C2362F and pSVHVWF-C2362A, respectively, are underlined. †(19). §Seq, sequencing of plasmid; Mut, mutagenesis reaction.

for 50 seconds and extension at 68°C for 24 minutes followed by final extension at 68°C for 7 minutes. The presence of the C2362F or C2362A mutation was monitored by restriction enzyme digestion of plasmid DNA with *Nsb*I (MBI Fermentas, St. Leon-Rot, Germany) or *Tau*I (MBI Fermentas), respectively. pSE280-*Hind*III-*Mun*I-C2362F or pSE280-*Hind*III-*Mun*I-C2362A were subsequently sequenced using CEQ 2000 Dye Terminator Cycle Sequencing (Beckman Coulter, Fullerton, CA, USA) and oligonucleotides 5 and 6 (Table II). The *Psh*AI-*Bgl*II fragment containing only the C2362F or C2362A mutation was transferred to pSE280-*Hind*III-*Mun*I-wt with compatible sites, creating pSE280-*Hind*III-*Mun*I-C2362F or pSE280-*Hind*III-*Mun*I-C2362A. Colonies containing either the C2362F or the C2362A plasmid were selected by restriction digestion of plasmid DNA with *Nsb*I or *Tau*I. The mutant *Hind*III-*Mun*I fragments were exchanged with the corresponding wt fragment in pSVHVWF creating pSVHVWF-C2362F and pSVHVWF-C2362A. All constructs were transformed and propagated in *E. coli* DH5α. DNA used in transient transfection experiments was purified with the plasmid maxi kit from Qiagen (Hilden, Germany) and the preparations used were screened by restriction digestion with *Nsb*I or *Tau*I.

Expression of recombinant VWF

Using the calcium phosphate precipitation method, 293T human kidney cells were cultured in T75 flasks and transiently transfected with a total of 27 μg plasmid DNA per flask (20). Cells were transfected with wt and pSVHVWF-C2362F (mutant) constructs alone or in combination to mimic the homozygous or heterozygous state in the patients. All transfections were performed essentially as previously described (19).

ADAMTS13-mediated proteolysis of VWF

Recombinant ADAMTS13 (rADAMTS13) was produced by transient transfection of the pcDNA3.1-ADAMTS13 V5/hisC expression vector (kindly provided by B.M. Luken and J. Voorberg, CLB research at Sanquin, Amsterdam, the Netherlands) in 293T human kidney cells. Conditioned media containing either recombinant VWF (rVWF) or rADAMTS13 were supplemented with Pefabloc SC (Roche Diagnostics,

Mannheim, Germany) to a final concentration of 1 mM and concentrated approximately 10 times by centrifugation in 5 mM Tris (pH 8.0) using Macrosep concentrators (cut-off value 100 kDa; Pall Gelman Laboratory, Ann Harbor, MI, USA). Protease activity of rADAMTS13 aliquots was determined with the immunoblotting method of Böhm and co-workers (21) using ADAMTS13 in pooled normal plasma as a standard. The sensitivity for the ADAMTS13 protease activity of rVWF: C2362F, wt/C2362F and wt, was assessed at a fixed concentration of the recombinant substrates and a 1:20 dilution of ADAMTS13 in 5 mM Tris pH 8.0. This dilution of rADAMTS13 protease showed approximately the same proteolytic activity as ADAMTS13 in normal pooled plasma. To mimic proteolysis of VWF during normal activity of ADAMTS13 this fixed amount of rADAMTS13 was used in the assay. The final concentration of urea in the reaction mixture was 0, 0.1, 0.5, 1.0 or 1.5 M. After 0, 8 and 24 h incubation at 37°C, aliquots were taken and the reaction was stopped by the addition of EDTA. Subsequently, the VWF multimeric distribution was analyzed. In all tests the same batch of concentrated rADAMTS13 was used.

Quantitative and qualitative analysis of VWF

In conditioned medium and cell lysate VWF:Ag was measured by ELISA (19) and the distribution of VWF multimers was assessed by nonreducing sodium dodecyl sulfate (SDS)-agarose gel electrophoresis on 1.5% gels essentially according to the protocol of Raines *et al.* (22). The subunit size of reduced recombinant or plasma VWF was analyzed on denaturing 5% SDS-polyacrylamide gel electrophoresis (PAGE) or on 3% MetaPhor® gels (BMA, Rockland, ME, USA). VWF was transferred to 0.45-µm Immobilon™-P polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA) by semi-dry blotting for SDS-PAGE gels and by capillary transfer for MetaPhor® gels. VWF was detected using the polyclonal anti-human VWF A082 antibody (Dako, Glostrup, Denmark) in combination with the peroxidase rabbit immunoglobulin-G ABC kit from Vectastain (PK-4001; Vector Laboratories, Burlingame, CA, USA). VWF was visualized with SuperSignal® West Pico chemoluminescent substrate kit from PIERCE (Perbio Science Nederland, Etten-Leur, the Netherlands) and/or precipitation colouring with 3,3'-diaminobenzidine (Sigma-Aldrich Chemie, Steinheim, Germany).

Results

Patients

The characteristics of one homozygous patient, one compound heterozygous patient, and one heterozygous carrier of the VWF mutation C2362F are listed in Table I. The data from the heterozygous carrier indicates a mainly quantitative defect with normal platelet dependent function of VWF (normal VWF:RCo/VWF:Ag and collagen binding activity (VWF:CB)/VWF:Ag ratios). The low levels of VWF-C2362F observed in the autosomal recessive severe VWD patients may be explained by several factors, *e.g.,* decreased synthesis of VWF, decreased secretion of VWF, or increased turn-over of mutant VWF due to conformational changes leading to elevated receptor-mediated clearance or increased susceptibility towards its natural protease, ADAMTS13. VWF and its propeptide are secreted in equimolar amounts and in steady state the VWF-propeptide and the mature VWF circulate at a distinct ratio. This was used to asses whether mutant VWF was cleared more rapidly than normal VWF. Under non-stimulated conditions the VWF-propeptide/VWF ratios for the homozygous, compound heterozygous and heterozygous subjects were, 0.07, 0.16 and 0.21, respectively, which was within the normal range (Table I) and did not indicate increased turn-over of VWF. In addition, *in vivo* clearance of VWF-C2362F was studied by determining the half-life of VWF as well as VWF-propeptide after infusion of DDAVP in a subject homozygous for the C2362F mutation. This resulted in a rapid six-fold increase in the level of VWF-propeptide compared with the basal level, while only a mild transient rise of VWF:Ag and a minimal rise in VWF:RCo occurred (Fig. 1 and Table I). FVIII:C activity increased 1.5-fold and remained >0.50 IU/mL for 4 hours. The half-life of VWF-C2362F was estimated at 3.6 h (Fig. 1A) indicating a two-fold decrease in its survival compared to normal VWF, while the half-life of 3.1 h for the VWF-propeptide remained normal. Although the level of VWF-C2362F was low in the homozygous subject, the mutant protein was detectable and could form both dimers and multimers (Fig. 1B). However, the VWF multimer analysis of plasma before and after DDAVP infusion also showed a remarkably high intensity smear of VWF. The smear in VWF multimers may indicate increased susceptibility of VWF-C2362F to proteolytic degradation, however mutations of cysteine residues have also been implicated in a multimer pattern with smear (23).

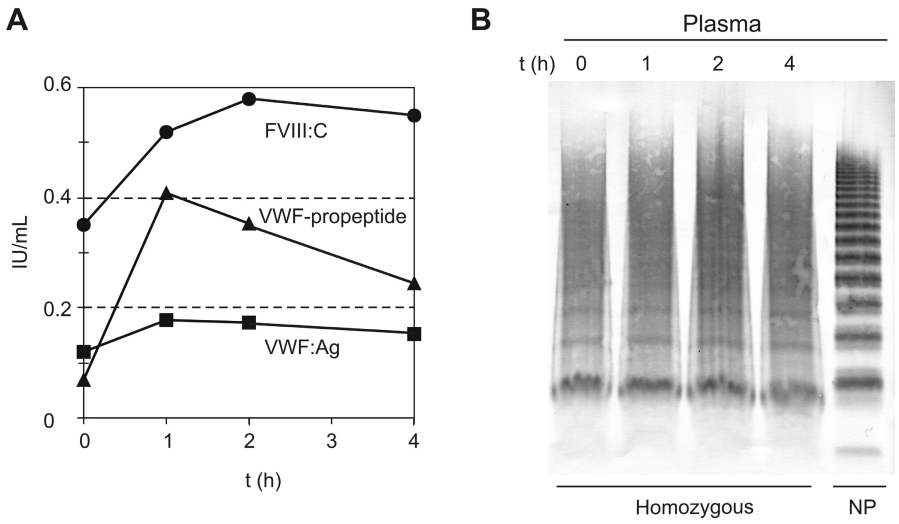

Fig. 1, FVIII:C, VWF:Ag and VWF-propeptide response after administration of DDAVP. Blood was collected before and after DDAVP infusion (0.3 µg/kg body weight) in the patient homozygous for the C2362F mutation (**A**). VWF multimer analysis (**B**) on 1.5% nonreducing SDS-agarose gel of plasma of the patient before and after DDAVP infusion (t = 0, 1, 2 and 4 hrs) compared with pooled normal plasma (NP). VWF-propeptide in U/mL.

Expression of recombinant VWF

Single transfections

The level and quality of rVWF-C2362F and rVWF-C2362A produced in both media and cell lysate were compared with that of rVWF-wt. Three independent experiments with duplicate single transfections of mutant and wt VWF constructs were performed . The VWF:Ag level of mutant rVWF-C2362F and rVWF-C2362A in conditioned medium was 8% and 12%, respectively, compared with that of rVWF-wt (Fig. 2). In cell lysates the levels of rVWF-C2362F and rVWF-C2362A were 275% and 308%, respectively, compared with those of rVWF-wt. The low level of secreted rVWF-C2362F is in accordance with the phenotype of the patient homozygous for this mutation (Table I) (10,12). The total production of rVWF (*i.e.*, the sum of rVWF in medium and lysate) was on average 2.4, 2.7 and 2.4 µg for rVWF: C2362F, C2362A and wt, respectively. Together, these data indicate normal production, but strong intracellular retention of rVWF-C2362F and rVWF-C2362A. The low levels of platelet VWF:Ag in the patients (Table I) suggests rapid intracellular degradation *in vivo* of the mutant protein. Mutant rVWF are secreted

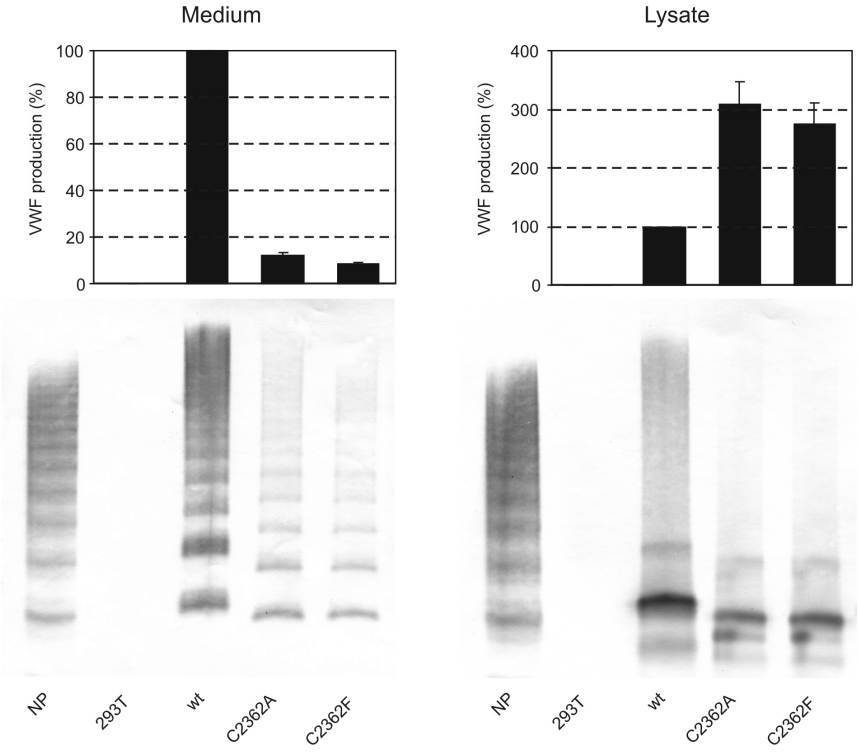

Fig. 2, single transfections of wt and mutant pSVHVWF constructs in 293T cells. The VWF production in medium (left panel) and lysate (right panel) is expressed relative to the amount of rVWF-wt protein. One hundred percent of rVWF-wt corresponds to 0.24-0.34 µg/mL in conditioned medium (5 mL) and 0.95-1.1 µg/mL in cell lysate (0.8 mL). Mean and standard deviation are based on six measurements (three independent experiments with two transfections in each experiment). The corresponding multimeric patterns are shown in the lower panel. The construct names are indicated below each lane. 293T corresponds to untransfected 293T cells. Pooled normal plasma (NP) was used as reference.

mainly as dimers and tetramers although higher molecular weight forms were present at a low level. Homodimers of rVWF-C2362F and rVWF-C2362A showed an increased electrophoretic mobility compared to rVWF-wt (Fig. 2).

Co-transfections

To study a possible dominant negative effect of the loss of cysteine 2362 on the dimerization and multimerization of normal VWF we performed co-transfections with a basal level of 3 µg wt construct, to which 1.5, 3, or 6 µg of mutant VWF construct or wt VWF construct was added. It was previously shown that in this

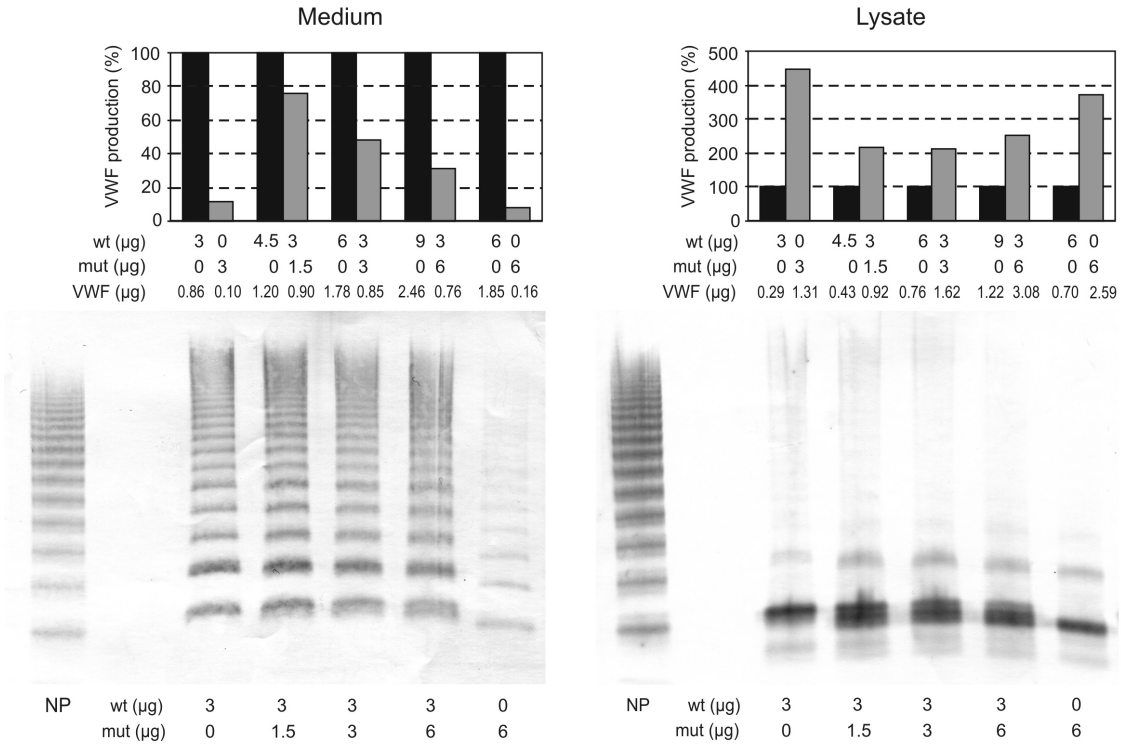

Fig. 3, co-transfections of wt and C2362F pSVHVWF constructs. The VWF production in medium (left panel) and lysate (right panel) is expressed for each co-transfection (grey bars) relative to the amount of rVWF-wt of the corresponding wt transfection (black bars). The amount of wt and mutant (mut) construct added in each transfection is indicated below the bars, as is the amount (µg) of rVWF produced in either medium or lysate. The amount of wt and mutant (mut) constructs in the corresponding transfections are indicated below the multimer blots. As reference, pooled normal plasma (NP) was used. Equal amounts of VWF were applied in each lane, except for the transfection in which only mutant construct was expressed as the expression level was too low to apply an equal amount.

experimental cell system the total amount of VWF produced in wt transfections increases linearly with the amount of DNA in the transfection and that the system is not overloaded by 9 µg of DNA (19). The results of these transfections are shown in Fig. 3. The total amount of rVWF produced was approximately the same in all co-transfections as in the corresponding wt transfections for both pSVHVWF-C2362F and pSVHVWF-C2362A. Increasing the amount of mutant construct included in the co-transfection resulted in a linear decrease of rVWF secreted in the medium. There was no indication of a dominant negative effect, given that a 50% reduction of rVWF secretion was observed in the 1:1

co-transfection of wt and mutant (Fig. 3). Also, the multimer pattern gave no indication for a dominant negative effect (Fig. 3). However, a clear difference in the electrophoretic mobility of VWF dimers was observed between wt and mutant VWF. A double band was detected in conditioned medium of the co-transfections, as well as in a heterozygous patient's plasma (Figs. 3 and 4A). Titration of wt construct with an increasing quantity of mutant construct resulted in an increasing intensity of the lower band while the upper band faded (Fig. 3). Although best visualized for dimers this was also seen for multimers. The upper band probably corresponds to homodimeric rVWF-wt, while the lower band corresponds to heterodimers of mutant and wt rVWF. The band of homodimeric rVWF-C2362F runs even faster (Fig. 3 left panel, right lane with mutant construct only). In the lysate three bands of dimeric rVWF were detected in close proximity of each other, which probably correspond to homodimeric wt, heterodimeric and homodimeric C2362F, respectively (Fig. 3, right panel). The same results were obtained in co-transfections of wt and the C2362A construct (data not shown).

VWF subunit size

Fig. 4A shows the increased electrophoretic mobility of both plasma and recombinant C2362F VWF as compared to normal plasma and recombinant wt VWF. The "heterozygous" wt/C2362F samples (both plasma and recombinant) show an intermediate VWF multimer pattern. These differences in electrophoretic mobility could be due to an altered conformation of the mutant protein or due to differences in the subunit size. The subunit size of VWF was analyzed on denaturing gels, after reduction of rVWF, and no difference in subunit size between wt, wt/C2362F or C2362F was observed in conditioned medium (Fig. 4B, left panel). The intracellular proVWF subunits were of equal size for both rVWF-wt and rVWF-C2362F. In addition, no difference in VWF subunit size was detected when rVWF was compared with VWF in patient plasma (Fig. 4B, right panel). Analysis of reduced rVWF in lysate did, however, show a distinct difference in processing of rVWF-C2362F and rVWF-wt (Fig. 4B, left panel). Both mature and proVWF were detected in lysate of wt transfections, but only proVWF was found in the lysates of cells transfected with the pSVHVWF-C2362F construct. This indicates that the majority of mutant rVWF-C2362F is not processed to mature VWF but is retained in the cell. As the subunit size of VWF-C2362F is

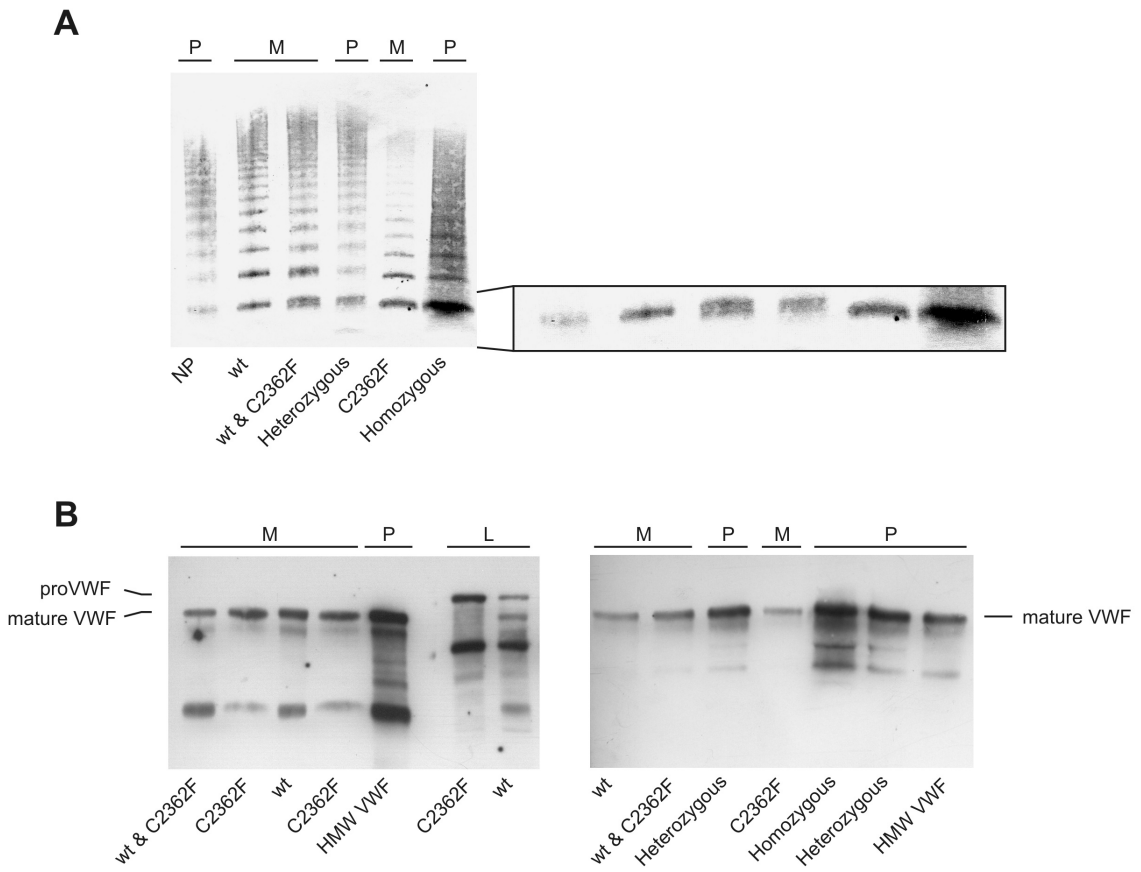

Fig. 4, multimer and subunit size of rVWF-wt and rVWF-C2362F. Non-reduced rVWF-wt (wt), rVWF-C2362F (C2362F), rVWF-wt/C2362F (wt & C2362F) and plasma of patients homozygous and heterozygous for the C2362F mutation were analyzed on SDS-agarose (1.5%) gel (**A**), and reduced VWF on SDS-PAGE (5%) gels (**B,** left) or on denaturing 3% MetaPhor gel (**B,** right) and immunoblotted with anti-VWF IgG. Patient plasma and recombinant mutant show faster migrating dimers and multimers (**A,** inset), but identical subunit size compared to normal VWF after reduction (**B**). M, medium; L, lysate; P, plasma; NP, pooled normal plasma; HMW VWF, purified high molecular weight VWF.

identical to normal VWF, the difference in electrophoretic mobility of homo- and heterodimers of VWF is probably best explained by an alternative conformation of the mutant subunit (Figs. 1B-4A).

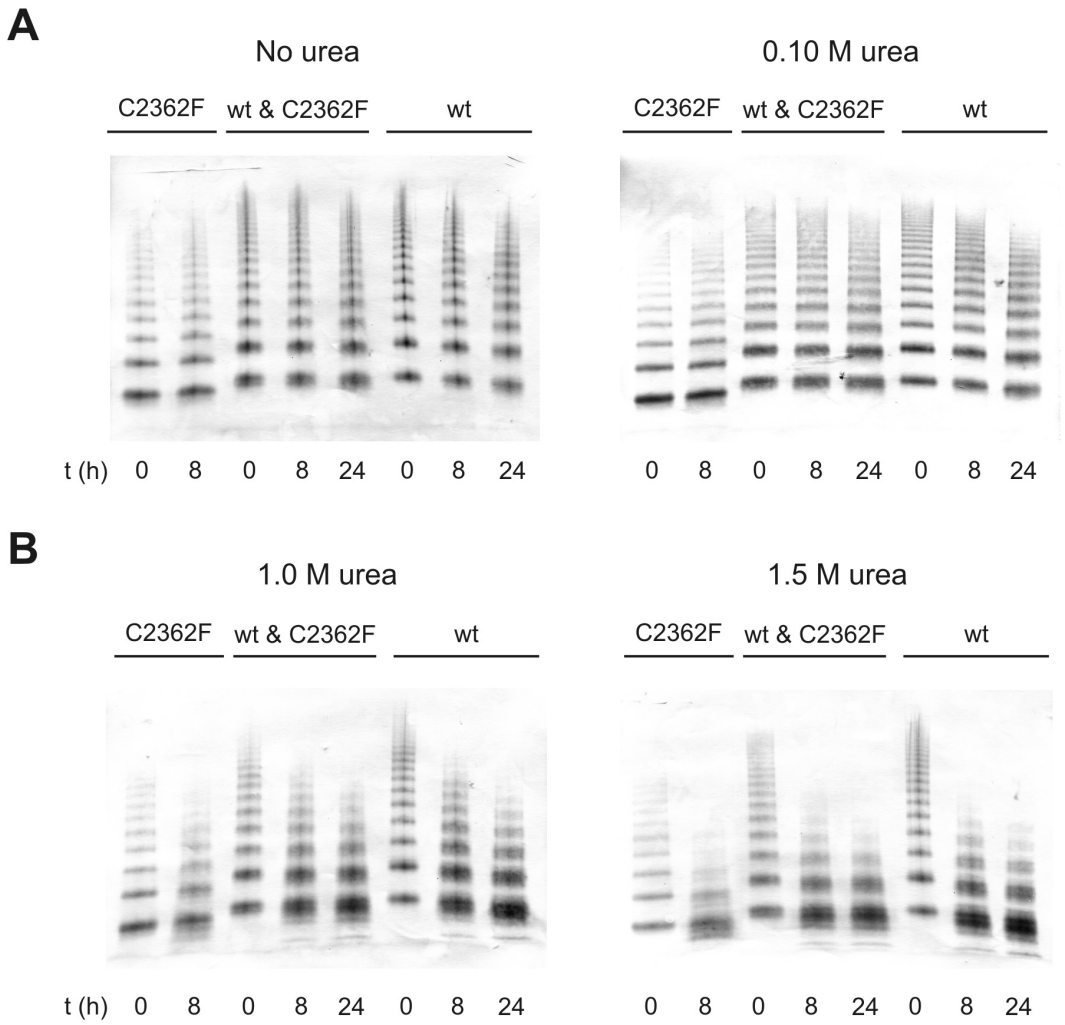

Fig. 5, ADAMTS13-mediated proteolysis of rVWF-wt and rVWF-C2362F. Recombinant VWF from single transfections of pSVHVWF-C2362F (C2362F), co-transfections (wt & C2362F) and single transfections of wt construct (wt) were incubated with recombinant ADAMTS13 for 0, 8 or 24 h (t) in the absence or presence of 0.1, 0.5, 1.0, 1.5 M urea (0.5 M urea not shown). The degree of proteolysis was assayed on SDS-agarose gel electrophoresis under non-reducing conditions.

ADAMTS13 proteolysis assay

After administration of DDAVP the half-life of mutant VWF-C2362F was found to be reduced (Fig. 1A). It has previously been shown by Castaman *et al.* (24) that plasma of patients homozygous for the C2362F mutation contains increased levels of degraded VWF. To investigate whether the reduced half-life of VWF-C2362F

was due to increased proteolysis of mutant VWF by ADAMTS13, proteolysis mediated by ADAMTS13 was studied in an *in vitro* assay. No or little degradation was detected in the absence or in the presence of 0.1 M urea (Fig. 5A). However, at 1.0 M or 1.5 M urea a gradual disappearance of the HMW multimers and the formation of the typical protease-dependent triplet structure was observed (Fig. 5B). Comparison of rVWF: wt, wt/C2362F and C2362F did not reveal any difference in the sensitivity of the rVWF substrates to cleavage by ADAMTS13.

Discussion

In patients with type 3 VWD, identification of the underlying mutations has been laborious since their location is not restricted to a certain region of the large VWF gene. The mutations that have been described mainly result in null alleles (5). However, some missense mutations of cysteines at the C-terminal region of VWF have been identified in type 3 VWD patients (6-9). We and others have previously studied mutated cysteines in the CK domain of VWF and have shown that mutation of cysteines involved in intrachain bonds result in a mainly quantitative VWF defect, while mutation of cysteines involved in interchain bonds result in a qualitative type 2A (subtype IID) VWF defect (Chapter 5) (7,19,25). The quantitative deficiency observed in the type 3 phenotype may be caused by the disruption of VWF monomer structure when an intrachain bond is lost, which subsequently leads to pronounced intracellular retention and has no dominant negative effect. On the contrary, the loss of an interchain bonds interferes with subsequent dimerization and multimerization and thus results in a dominant negative defect which corresponds with the type 2A(IID) phenotype. The present study, however, investigated the effects of a mutation of a cysteine (C2362F) located outside the CK domain. This location predicts the cysteine to be involved in an intrachain bond and, to date, no specific function has been assigned to this region of VWF. The C2362F mutation is found in patients with a severe quantitative VWF defect. Based on this severe quantitative autosomal recessive VWD phenotype and our previous studies (19), it was hypothesized that this mutation also would result in intracellular retention without a dominant negative effect. Patient data indicated a severe VWF deficiency in homozygous (C2362F) and compound heterozygous (C2362F/null allele) patients, whereas VWF was reduced by about 50% with normal platelet-dependent function in heterozygous

carriers of the C2362F mutation (Table I). Expression of recombinant VWF-C2362F showed normal production, but nearly complete intracellular retention (Fig. 2). No sign of a dominant negative effect was found in co-transfections, indicating that the ultimate effects of the C2362F mutation are similar to those of a null allele (Fig. 3). Expression of the alternate mutant C2362A gave similar results indicating that monomer conformation of VWF is affected by the loss of the cysteine rather than by the introduction of phenylalanine. The processing of intracellularly retained rVWF-C2362F is severely hampered as only proVWF is present in lysate (Fig. 4B, left panel). This was in agreement with the low VWF-propeptide levels observed in patients plasma (Table I), which indicates that there is no selective retention of mature VWF, but retention of VWF-propeptide as well (probably as unprocessed proVWF). In addition, the low platelet VWF:Ag observed in the patients (Table I) indicates intracellular degradation. The abnormal electrophoretic mobility observed for both the phenylalanine and the alanine mutant is probably due to a conformational change as the subunit size of reduced VWF-C2362F was identical to that of rVWF-wt (Fig. 4). The presence of a smear in the plasma VWF multimer pattern of the patient homozygous for VWF-C2362F (Fig. 1B), the absence of this smear in the multimer pattern of rVWF-C2362F, the previous finding of increased concentrations of proteolytic fragments of VWF-C2362F in patient's plasma (24), as well as the reduced half-life of VWF-C2362F after DDAVP infusion (Fig. 1A), may all result from increased degradation of VWF-C2362F in plasma, elevated receptor-mediated clearance of mutant VWF, or a combination thereof. The increased presence of proteolytic fragments in patients plasma observed by Castaman and co-workers is however not explained by an increased sensitivity of the mutant protein to proteolysis by rADAMTS13 (Fig. 5). Additional proteases, which were not present in our ADAMTS13 preparation, in combination with ADAMTS13 may be responsible for the observed proteolysis. However, considering the very low secretion of rVWF-C2362F, the contribution of *in vivo* degradation of VWF-C2362F in plasma to the overall reduction of VWF in the homozygous patients seems to be limited. To conclude, this study has shown that the C2362F mutation causes a severe quantitative defect. We have demonstrated that the autosomal recessive severe VWD phenotype is the result of impaired secretion due to strong intracellular retention of unprocessed mutant proVWF subunits, which is caused by the loss of an intrachain bond and not by the introduction of an bulky amino acid side chain.

Acknowledgements

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Differential effects of the loss of intrachain *versus* interchain bonds in the cystine knot domain of von Willebrand factor on the clinical phenotype of von Willebrand disease

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Abstract

Von Willebrand factor (VWF) contains a large number of cysteine residues, which all participate in the formation of disulfide bonds. Mutations of cysteine residues in the cystine knot (CK) domain of VWF resulting in dimerization defects have been identified in both qualitative type 2A (former subtype IID, 2A(IID)) and quantitative type 3 von Willebrand disease (VWD). We hypothesized that this difference in phenotype is related to whether the mutated cysteine residue is involved in either interchain or intrachain disulfide bond formation. To test this hypothesis we studied three mutations in the CK domain of VWF. We compared the effects of the novel C2773S mutation, which was identified in a family with type 2A(IID) VWD, with those of the C2739Y and C2754W mutations, which were previously identified in patients with type 3 VWD. The effect of these mutations on dimerization and multimerization of VWF was studied by transient expression in 293T cells. Co-transfection of wild-type (wt) and C2773S VWF constructs reproduced the plasma phenotype of heterozygous type 2A(IID) patients, with normal to high levels of VWF antigen (VWF:Ag), absence of high molecular weight multimers, and the presence of intervening bands between the normal multimers. In contrast, single transfections of C2739Y or C2754W, resulted in a quantitative VWF defect with low VWF:Ag levels, and a 50% reduction of VWF:Ag in co-transfections of wt and mutant constructs and only a minor effect on VWF multimerization. We showed preferential N-terminal dimerization of VWF-C2773S and both C- and N-terminal dimerization of VWF-C2754W. Our data suggest that loss of a single disulfide bond in the CK domain of VWF will lead to a recessive, quantitative VWF deficiency if an intrachain disulfide bond is involved, and to a dominant negative, qualitative defect of VWF if an interchain disulfide bond is involved.

Introduction

Von Willebrand factor (VWF) is a high molecular weight (HMW) plasma glycoprotein with two vital functions. At the site of vascular injury, VWF mediates the adhesion of platelets to the vessel wall and initiates the formation of a platelet plug by aggregation of platelets. In addition, VWF is indirectly involved in secondary hemostasis as the carrier protein of coagulation factor VIII (FVIII) with which it forms a non-covalent complex. The function of VWF depends on the proper formation of HMW multimers, which range from 0.5 to 10 million Daltons in mass. The multimerization process is very important as the highest molecular weight multimers are most effective in supporting platelet adhesion and aggregation. Formation of VWF multimers starts with dimerization of proVWF monomers in the endoplasmic reticulum (ER) (1,2). ProVWF monomers are linked pairwise through interchain disulfide bond(s) at the C-terminal cystine knot (CK) domain (3). Subsequently, multimerization occurs in the Golgi apparatus, where proVWF dimers are covalently linked by interchain disulfide bonds at the N-terminal end of the VWF molecule with concurrent removal of the VWF-propeptide. The resulting multimers consist of an even number of mature VWF subunits (1,2). VWF contains a large number of cysteine residues (8.2%) that all form interchain or intrachain disulfide bonds and are of great importance for the structural integrity of the VWF monomer and the processes of di- and multimerization (4).

Alterations in the VWF gene sequence resulting in a modification of the level and/or function of the VWF protein may cause von Willebrand disease (VWD) (5), which is the most common inherited bleeding disorder. Missense mutations of cysteines located in the CK domain have been found both in patients with quantitative and qualitative VWF defects. In patients with a virtually complete absence of VWF, type 3 VWD, alterations of cysteine residues 2739 (6), 2754 (7), 2804 (8) and 2806 (9) have been described. However, in the same region changes of cysteine residues at position 2771 (10) and 2773 (11) result in type 2A (former subtype IID, 2A(IID)) VWD, which is characterized by a qualitative, multimerization defect. We hypothesized that the differences in phenotype among patients with mutations at cysteine residues in the CK domain depend on whether the mutated cysteine residue participates in an interchain or intrachain disulfide bond.

To investigate this hypothesis we have screened a type 2A(IID) VWD family for a mutation in the CK domain, anticipating that this would be a mutation at a cysteine residue involved in the formation of an interchain disulfide bond. Indeed, we identified such a mutation in the CK domain (C2773S). We examined the effect of this novel missense mutation at the level of dimerization and multimerization by transient expression of the recombinant protein and compared it with the effects of two type 3 VWD mutations in the CK domain, C2739Y (6) and C2754W (7).

Materials and methods

Patients

The proposita (III-5; Table I) has a severe bleeding tendency, manifested in spontaneous bruises, and bruises of abnormal size after mild trauma. She suffered from frequent epistaxes during childhood, heavy menorrhagia causing anaemia, postpartum hemorrhage requiring blood transfusion, and prolonged bleeding after tooth extraction. The daughter (IV-1) has a severe bleeding tendency with frequent epistaxes (once a week), abnormal bruising and heavy menorrhagia. At the age of 4 she experienced gastrointestinal bleeding. A son (IV-2) died after tonsillectomy. The remaining family members, the father (III-4), daughter (IV-3) and son (IV-4) are not affected. Patients' plasma was analyzed for factor VIII activity (FVIII:C), VWF antigen (VWF:Ag) and VWF ristocetin cofactor activity (VWF:RCo). The bleeding time was determined according to the Ivy method using a micro lancet. The phenotypic characteristics of affected and unaffected family members are presented in Table I. The affected mother (III-5) and daughter (IV-1) were diagnosed as type 2A(IID) VWD based on the prolonged bleeding time, normal levels of VWF:Ag, strongly reduced VWF:RCo (Table I), and the typical VWF multimer pattern, lacking HMW multimers and including aberrant, odd-numbered multimers between the normal VWF multimers (Fig. 1B). The pedigree (Fig. 1A) shows dominant inheritance with transmission from the affected mother to the affected daughter and possibly the deceased son (IV-2). For comparative studies we used two previously described type 3 VWD mutations identified in patients compound heterozygous for the C2739Y mutation and a null allele (6) and homozygous for the C2754W mutation (7).

Table I: Phenotypic characteristics

Patient Nb	Sex (f/m)	BT Ivy (min)	FVIII:C (U/mL)	VWF:Ag (U/mL)	VWF:RCo (U/mL)	Blood group	Bleeding tendency
III-4	m	2.75	2.0	1.8	1.9	A	None
III-5	f	>15	0.98	1.92	<0.20	O	Severe
IV-1	f	>15	0.74	1.07	<0.20	O	Severe
IV-2	m	na	na	na	na	na	Severe*
IV-3	f	3.75	1.50	0.92	1.06	O	None
IV-4	m	2.5	1.60	1.36	1.34	A	None
Normal		0-4	0.5-2	0.4-2	0.5-2		

f, female. m, male. BT, bleeding time. na, not available. *Died after tonsillectomy.

Screening for mutations

Based on the type 2A(IID) VWD phenotype of the patients, we anticipated a mutation to be located in the CK domain of VWF. Thus, exons 51 and 52 of the VWF gene of patients III-5, IV-1, the unaffected father III-4 and an unrelated healthy person were amplified using oligonucleotides 1 and 2 for exon 51 and oligonucleotides 3 and 4 for exon 52 (Table II). Both strands were sequenced by ABI-prism Big-Dye™ terminator cycle sequencing (PE Applied Biosystems, Nieuwerkerk a/d IJssel, the Netherlands).

Plasmid construction

Construction of plasmids encoding VWF-wt, VWF-C2739Y and VWF-C2754W was described before (12). The C2773S mutation was introduced in the pSVHVWF plasmid containing the full-length wt cDNA of VWF via site-directed mutagenesis utilizing oligonucleotides 5 and 6 (Table II) and the QuikChangeXL™ site-directed mutagenesis kit from Stratagene (La Jolla, CA, USA). Successful incorporation of the mutation was verified by restriction digestion with *Mnl*I of the colony-PCR

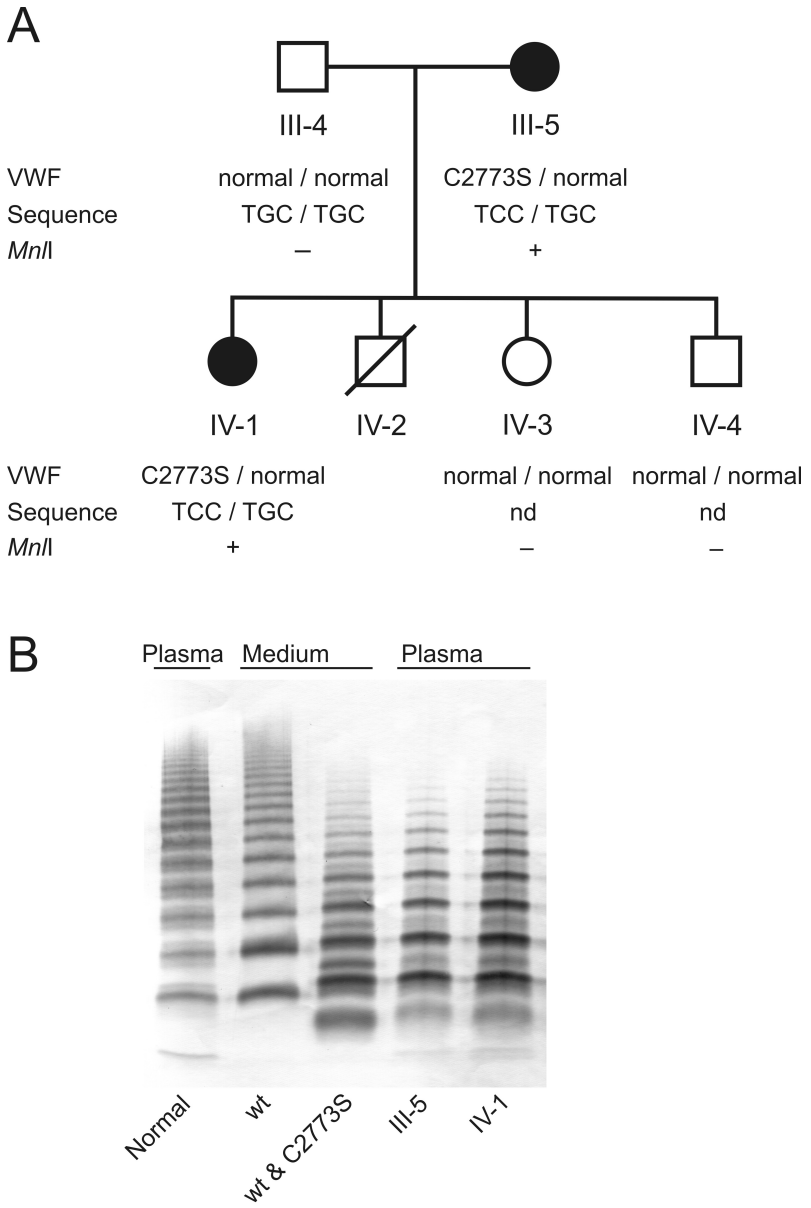

Fig. 1, sequence and multimer data of the family with 2A(IID) VWD. Pedigree showing the presence of the C2773S mutation (**A**) determined by sequencing (codon indicated) and by *Mnl*I digestion (mutation present +, absent -). Individual IV-2 is deceased and no samples were available for analysis. nd, not determined; open symbol, unaffected individual; filled symbol, affected individual; square, male; circle, female. Analysis of VWF multimers (**B**) on non-reducing SDS-agarose (1.5%) gel. Shown are pooled normal plasma (normal), rVWF-wt (wt), recombinant co-expressed VWF (wt & C2773S), and plasma from the mother (III-5) and daughter (IV-1). Note the absence of HMW multimers and the intervening bands representing multimers with an odd-number of VWF subunits appearing between the normal, even, multimers both in recombinant and patient plasma. This pattern is typical of type 2A(IID) VWD.

Table II: Oligonucleotides for sequencing and mutagenesis

Oligonucleotide		Sequence*	Direction†	Overlapping region	Purpose***
Nb	Name	(5'to 3')	(F/R)	(chromosomal/plasmid)	
1	51A	GGCTGAAGAGTGTTCTCTAG	F	38/36-55§	Seq genomic
2	51B	ATAGGTATCCGAACACGG	R	38/261-244§	Seq genomic
3	52A	TGCCTAAGCCAGGACTTCCC	F	38/721-740§	Seq genomic
4	52B	GGCCATCAGGGCAAGGCAGG	R	38/1020-1001§	Seq genomic
5	C2773S	GGACCAGTGCTCCTcCTGCTCTCCGACACG	F	8574-8603**	Mutagenesis
6	C2773S	CGTGTCGGAGAGCAGgAGGAGCACTGGTCC	R	8603-8574**	Mutagenesis
7	pSVHVWF-C2773S	CTGCTGTGACACATGTGAGGAGC	F	8412-8434**	Seq plasmid Colony PCR
8	pSVHVWF-C2773S	GAGCTCAGCCTTTATTGTGGGC	R	8837-8816**	Seq plasmid Colony PCR
9	pSVHVWF-C2773S	CAGGGTTATTGTCTCATGAGCGG	R	9278-9256**	Seq plasmid Colony PCR
10	pcDNA3.1-ADAMTS13 V5/hisC	TAT*AAGCTT*GGATGCACCAGCGT	F	443-456††	Signal peptide
11	pcDNA3.1-ADAMTS13 V5/hisC	TATATCTCCTGGTCCAGTCGTAC	R	977-955††	Signal peptide

*Nucleotide substitution indicated in lower csae, *Mnl*I recognition sequence underlined, *Hind*III restriction site in italic. †F, forward; R, reverse. §Numbering according to Mancuso *et al.* (13). **Numbering according to pSVHVWF1 construct previously described in Tjernberg *et al.* (12). ††Numbering according to Genbank sequence NM_139025 of ADAMTS13. ***Seq genomic, sequencing of genomic DNA; Mutagenesis, introduction of missense mutation; Seq plasmid, sequencing of plasmid DNA; Colony PCR, screening of transformed *E. coli* by PCR and subsequent *Mnl*I restriction digestion; Signal peptide, introduction of ADAMTS13 signal-peptide by PCR.

products obtained with oligonucleotide 7 in combination with oligonucleotide 8 or 9 (Table II). Subsequently, plasmid DNA was prepared and directly sequenced on the ABI 310 using the ABI-Prism Big-Dye™ terminator cycle-sequencing kit and oligonucleotides 7 to 9. DNA containing the C2773S mutation only was digested with *Eco*RV and *Mun*I and the mutant fragment was ligated into pSVHVWF with compatible sites, creating pSVHVWF-C2773S. Colonies containing the pSVHVWF-C2773S plasmid were selected by restriction digestion with *Mnl*I of the colony PCR products and the DNA was sequenced (see above).

Full-length ADAMTS13 cDNA was cloned from human liver cDNA essentially as described previously (14). In the present construct, the signal-peptide of ADAMTS13 was introduced via a PCR fragment generated with oligonucleotides 10 and 11 (Table II). This fragment replaced the original sequence from the pMIB V5/hisC ADAMTS13 vector up to the first internal *Bam*HI site. The resulting full-length ADAMTS13 sequence is identical to Genbank sequence NM_139025 and was cloned into the *Hind*III and *Xho*I restriction sites of pcDNA3.1 generating pcDNA3.1-ADAMTS13 V5/hisC. All constructs were transformed and propagated in *E. coli* DH5α. DNA used in transient transfection experiments was purified with the plasmid maxi kit from Qiagen (Hilden, Germany) and the preparations of pSVHVWF-C2773S were screened with *Mnl*I and sequenced as described.

Expression of recombinant VWF

293T human kidney cells were transiently transfected with pSVHVWF-wt, pSVHVWF-C2739Y, pSVHVWF-C2754W and pSVHVWF-C2773S plasmids in single transfections and with pSVHVWF-wt and pSVHVWF-C2773S in co-transfections using the calcium phosphate precipitation method. Transfections and collection of conditioned medium and cell lysate were as previously described (12).

N-terminal dimerization assay utilizing ADAMTS13-mediated proteolysis of VWF

Recombinant ADAMTS13 (rADAMTS13) was produced by transient transfection of the pcDNA3.1-ADAMTS13 V5/hisC expression vector. 293T cells were cultured in T75 flasks and transfected with a total of 27 µg DNA per flask. The pSVHVWF-C2754W plasmid was used in single transfections while the wt and C2773S constructs were transfected alone or in combination at a ratio of 1:1 to

mimic the heterozygous state in patients. All transfections were essentially performed as previously described (12). Conditioned medium was collected and Pefabloc SC (Roche Diagnostics, Mannheim, Germany) was added to a final concentration of 1 mM. The medium containing either recombinant VWF (rVWF) or rADAMTS13 was concentrated ~10 times by centrifugation in 5 mM Tris (pH 8.0) using Macrosep concentrators (cut-off value 100 kDa, Pall Gellman Laboratory, Ann Harbor, MI, USA). Protease activity in rADAMTS13 aliquots was determined according to the immunoblotting method of Böhm *et al.* (15) using ADAMTS13 in pooled normal plasma as a standard. Recombinant VWF substrates (wt, wt co-expressed with C2773S, C2773S and C2754W) were incubated at 37°C for 24 hrs with rADAMTS13 diluted 1:5 in 5 mM Tris pH 8.0 and 1.5 M urea. Proteolysis was stopped by addition of EDTA. In all experiments the same batch of concentrated rADAMTS13, shown to be as active as ADAMTS13 in pooled normal plasma, was used. The distribution of VWF fragments after ADAMTS13-mediated proteolysis was analyzed on denaturing 5% PAGE gels. Proteins were transferred to a PVDF membrane by semi-dry blotting and the membrane was incubated with Western Blocking Reagent from Roche Diagnostics (Mannheim, Germany). VWF was detected with a primary polyclonal anti-human VWF antibody (A082, Dako, Glostrup, Denmark). Monoclonal antibodies directed against human VWF domain A1 (MoAb 723) and domain A3 (MoAb 400) (16) were a generous gift from Dr. D. Meyer (INSERM U143, Le Kremlin-Bicêtre, France). Horseradish peroxidase (HRP) conjugated goat anti-rabbit or anti-mouse IgG were from Bio-Rad (Veenendaal, the Netherlands).

Quantitative and qualitative analysis of VWF

In conditioned media and cell lysates, VWF:Ag levels were measured by ELISA (12) and VWF multimerization was assessed on non-reducing SDS-agarose (1.5%) gels essentially according to the protocol of Raines *et al.* (17). VWF multimers were detected using a polyclonal anti-human VWF antibody (A082, Dako) in combination with the Peroxidase Rabbit IgG ABC kit from Vectastain (PK-4001, Vector Laboratories, Burlingame, CA, USA). VWF was visualized with SuperSignal® West Pico chemoluminescent substrate kit from PIERCE (Perbio Science Nederland, Etten-Leur, NL) and/or precipitation colouring with 3,3'-diamino-benzidine (DAB, Sigma-Aldrich Chemie, Steinheim, Germany).

Results

Screening for mutations

The 2A(IID) phenotype suggested the presence of a mutation in the CK domain and sequencing of exon 52 showed a previously unreported transversion of a guanine to cytosine, 8318G>C, resulting in the change of cysteine 2773 to serine (C2773S, nomenclature according to reference (18)). Heterozygosity for this mutation was confirmed by the presence of an additional *Mnl*I restriction site (Fig. 1A).

Expression of recombinant VWF

Single transfections

The effect of the mutations C2773S (type 2A(IID)), C2739Y (type 3) and C2754W (type 3) on the quantity and the quality of recombinant VWF was studied in transient single transfection experiments. Two independent experiments were performed in triplicate for mutant and wt VWF constructs. The VWF:Ag level of rVWF-C2773S in conditioned medium was 138% compared with that of rVWF-wt, indicating a normal to high level of production and secretion of rVWF-C2773S (Fig. 2). However, multimer analysis revealed a complete lack of high and intermediate molecular weight multimers and excess of monomeric and dimeric rVWF-C2773S. The multimer analysis, of both conditioned media and cell lysates, further revealed a difference in the electrophoretic mobility of homodimeric rVWF-C2773S compared with homodimers of rVWF-wt and homodimers of rVWF-C2739Y or rVWF-C2754W. The levels of secreted rVWF-C2739Y and rVWF-C2754W were very low (0.5% and 5% respectively), which is in accordance with the type 3 VWD phenotype of the patients described with those mutations (6,7). The intracellular levels of rVWF-wt, rVWF-C2773S and rVWF-C2754W were similar (100%, 113% and 116% respectively), whereas the intracellular level of rVWF-C2739Y was significantly reduced (45%).

Co-transfections

The effect of the VWF-C2773S subunit on the dimerization and multimerization of VWF-wt was studied in co-transfections, in which 1.5, 3, or 6 µg of mutant or wt

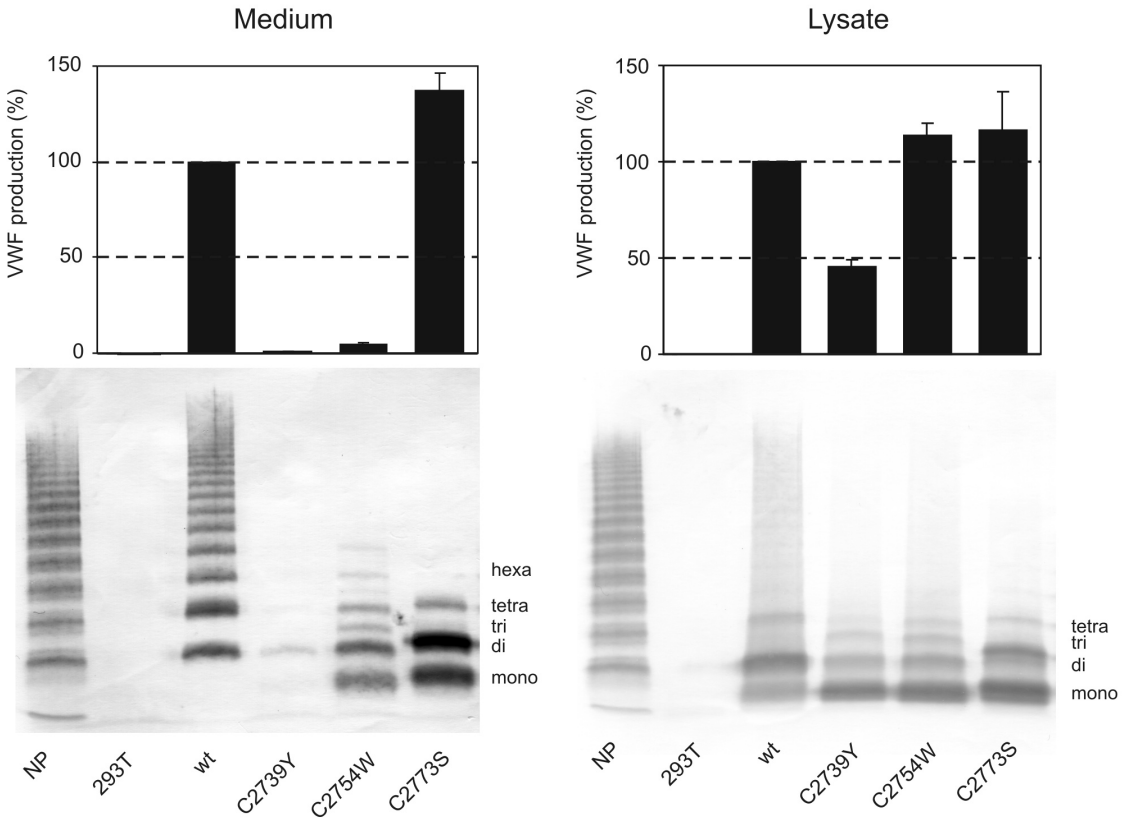

Fig. 2, single transfections of wt and mutant pSVHVWF constructs in 293T cells. The production of mutant VWF in medium (left panel) and lysate (right panel) is expressed relative to that of rVWF-wt. One hundred percent of wt-VWF corresponds to 0.41-0.70 µg/mL in conditioned medium and 1.3-1.8 µg/mL in cell lysate. Mean and standard deviation are based on six measurements (two independent experiments with three transfections in each experiment). The corresponding multimeric patterns are shown in the lower panel. The construct names are indicated below each lane. 293T corresponds to untransfected 293T cells. Pooled normal plasma (NP) was used as reference. Oligomers are indicated.

VWF construct was added to 3 µg wt construct. We previously showed that in our experimental cell system the total amount of VWF produced in wt transfections increases linearly with the amount of DNA in the transfection and that the system is not overloaded by 9 µg of DNA (12). The results are shown in Fig 3. The total amount of rVWF produced (sum of rVWF in medium and lysate) was in all cases higher in the co-transfections (2.7-6.8 µg) than in the corresponding wt transfections (2.1-4.3 µg). A relative increase from 135% to 181% of secreted rVWF, and a slight increase from 99% to 110% of retained rVWF was detected when the

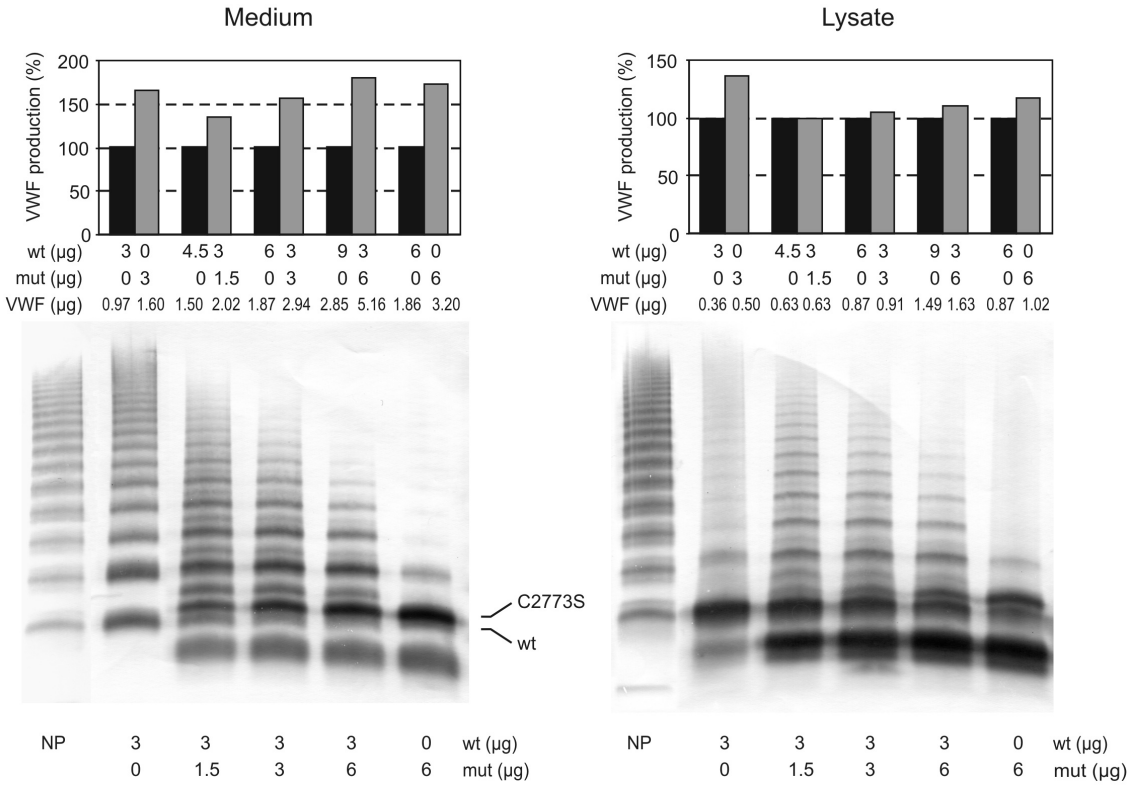

Fig. 3, co-transfections of wt and pSVHVWF-C2773S constructs. The VWF production in medium (left panel) and lysate (right panel) is expressed for each co-transfection (grey bars) relative to that of the corresponding wt transfection (black bars). The amount of wt and mutant (mut) construct added in each transfection is indicated below the bars as is the total amount (µg) of VWF produced in either medium or lysate. Below the multimer blots the amounts of wt and mutant (mut) construct in the corresponding transfections are indicated. Pooled normal plasma (NP) was used as a reference. Equal amounts of VWF were applied in each lane. The origin of the two populations of dimer bands is indicated as wt and C2773S.

amount of pSVHVWF-C2773S construct in the transfections was increased. The co-transfection of mutant and wt VWF construct at a ratio of 1:1 clearly reproduced the phenotype of the patients: normal to high levels of VWF:Ag, lack of HMW multimers and odd-numbered multimers appearing between normal VWF multimers (Table I and Figs. 1B and 3). When more mutant construct was included in the co-transfection, a switch from intermediate to low molecular weight (LMW) multimers occurred, indicating hampered multimerization. Furthermore, a clear difference in the electrophoretic mobility of rVWF-wt,

rVWF-wt co-expressed with rVWF-C2773S and rVWF-C2773S was detected for dimeric VWF. Two populations of dimeric VWF molecules are present in the patient's plasma and in the media of co-transfections (Figs. 1B and 3). The upper band probably corresponds to dimers formed through dimerization at the N-terminus of VWF whereas the lower band corresponds to dimers formed through normal dimerization at the C-terminus of VWF. Increasing the ratio of mutant over wt in the co-transfection leads to an increase in the intensity of the upper band whereas that of the lower band decreases. This suggests that the bulk of rVWF-C2773S dimers are formed through N-terminal dimerization whereas rVWF-wt dimers are formed through normal dimerization at the C-terminus of VWF.

Analysis of N- and C-terminal dimerization of VWF

Defective dimerization and formation of dimers via the N-terminal pairing is suggested by the observation of excess of monomers and dimers in media of cells transfected with pSVHVWF-C2773S. We aimed to demonstrate the presence of N-terminal dimerization and the lack of C-terminal pairing. ADAMTS13 protease cleaves VWF in the A2 domain between aa 1605 and 1606, which enables separate detection of N- and C-terminal VWF fragments. To identify N- and C-terminal VWF fragments, we utilized monoclonal antibodies 723 and 400, which were raised against the A1 and A3 domain of VWF, respectively (16). In theory, cleavage of VWF-wt and VWF-C2773S by ADAMTS13 are expected to yield the fragments depicted in Fig. 4A. After proteolysis by ADAMTS13 VWF fragments were separated on denaturing gels both under non-reduced and reduced conditions and N- and C-terminal VWF fragments were detected by immunoblotting using the A1 and A3 specific monoclonal antibodies (Fig. 4B). ADAMTS13 proteolysis of normal C-terminally dimerized and N-terminally multimerized VWF should yield dimeric VWF fragments linked at either the C- or N-terminus of VWF, designated Cd and Nd, respectively (Fig. 4A).

Proteolysis of HMW VWF purified from normal plasma and rVWF-wt indeed resulted in formation of Cd and Nd, indicating intact dimerization and multimerization of VWF (Fig. 4B). Alteration of C2773 may seriously affect the ability of VWF to form C-terminal dimers in the ER resulting in an excess of monomeric VWF and possibly N-terminally formed dimers as depicted in Fig. 4A.

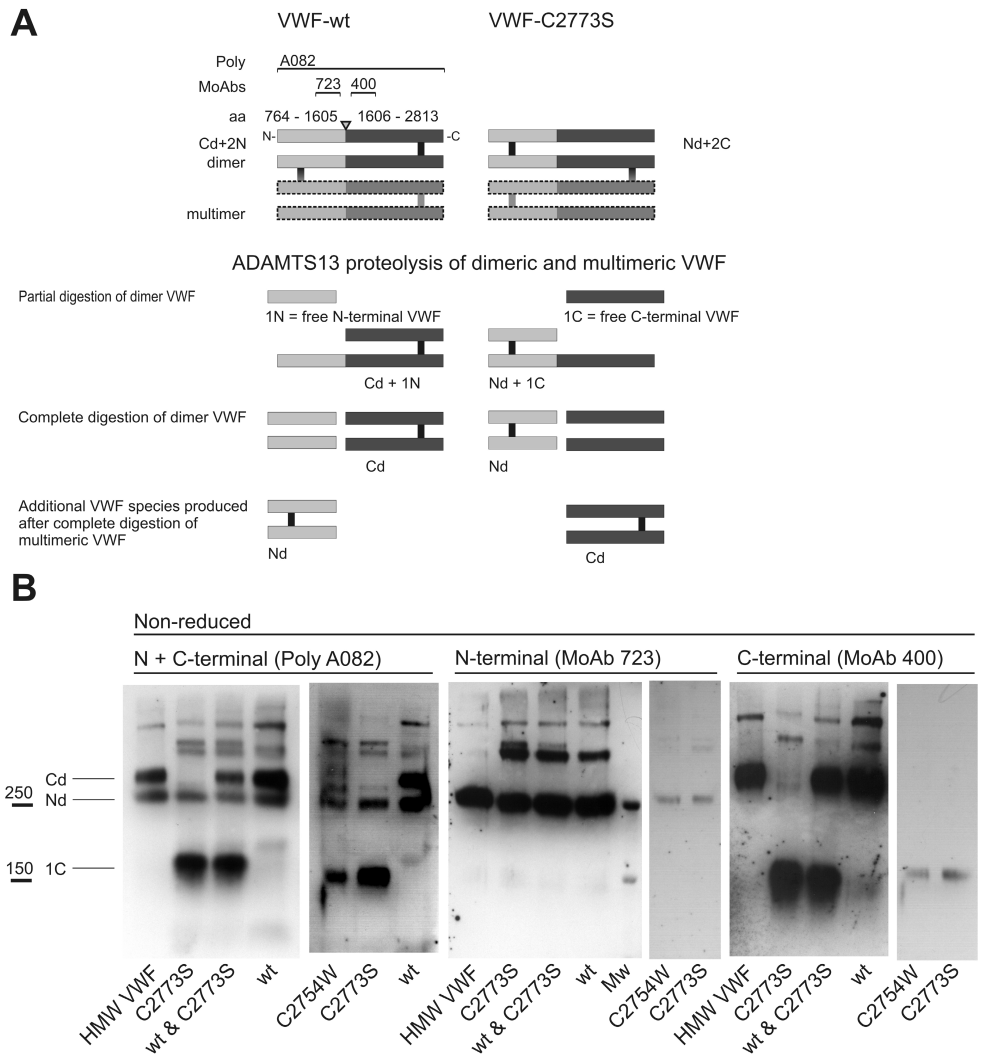

Fig. 4, analysis of N- and C-terminal dimerization of VWF. A schematic overview of expected proteolysis products (dimers and smaller) after ADAMTS13 digestion of VWF-wt and VWF-C2773S dimers and multimers are shown in panel **A**. The site of proteolysis is depicted with a triangle and lies between the recognition sites in the A1 and the A3 region for the two monoclonal antibodies 723 and 400, respectively. This enables detection of proteolysis products with the N-terminal (aa 764-1605, 1N) or C-terminal (aa 1606-2813, 1C) part of VWF. VWF was also detected with polyclonal antibody A082. Dimers formed through disulfide bonding at the C- or N-terminus of VWF are designated Cd+2N and Nd+2C. Complete ADAMTS13 digestion of VWF results in C-terminal dimers (Cd) and/or N-terminal dimers (Nd). The composition of non-reduced recombinant VWF of C2773S, co-expressed (1:1 ratio of wt and C2773S), wt, C2754W and high molecular weight VWF (HMW VWF) purified from plasma after 24 h of ADAMTS13-mediated proteolysis was studied by separation on four different 5% SDS-PAGE gels (**B**). Reduction of VWF samples resulted in detection of the mature VWF subunit, N-terminal and C-terminal fragments of VWF (results not shown). Molecular marker (Mw, is shown in lane 12) and is depicted on the left in the figure. Bands corresponding to 250 kD and 150 kD are indicated.

Proteolysis of monomers and N-terminally formed dimers would then yield an excess of free C-terminal VWF (1C) and Nd. Indeed, proteolysis of rVWF-C2773S resulted in main bands corresponding to N-terminally formed dimers and free C-terminal which were detected with MoAbs 723 and 400, respectively (Fig. 4B). Further, normal C-terminally linked (Cd) rVWF-C2773S was detected only as a faint band with MoAb 400 confirming that alteration of cysteine 2773 results in a dimerization defect. Proteolysis of co-expressed rVWF-wt and rVWF-C2773S (at a ratio of 1:1) resulted in detection of both normal C-terminal dimerization (Cd) and N-terminal multimerization (Nd) similar to rVWF-wt. Additionally, excess of free C-terminal fragments (1C) is detected indicating N-terminal dimerization similar as observed for rVWF-C2773S (Fig. 4B). These results may be explained by the segregation of dimerization and multimerization of VWF to different compartments in the cell. While C-terminal dimerization of rVWF-wt remains intact and generates normal dimers in the ER most C2773S will be monomeric due to its defective dimerization. Therefore it is unlikely that C2773S interacts with either wt or mutant VWF subunits (Figs. 3 and 4B) in the ER. In the Golgi wt dimers and mutant monomers both participate in multimerization leading to the addition of mutant monomers to wt multimers hampering further multimerization of wt multimers but also N-terminal dimerization of mutant monomers (Fig. 3).

ADAMTS13 digestion of rVWF-C2754W also resulted in Nd and free C-terminal VWF (1C) as the main end products (Fig. 4B). The intensity of Cd, Nd and free C-terminal VWF bands were assessed by densitometric analysis of the second blot in Fig. 4B (VWF detected with polyclonal antibody A082). The ratio of Nd *vs* Cd VWF was 1:0.63 for C2754W and 1:0.15 for C2773S. This indicates a four-fold higher level of relative residual C-terminal pairing for VWF-C2754W compared with the very low level observed for VWF-C2773S, which may explain the detection of octamers in single transfections of C2754W and only tetramers for C2773S. Further, a 1.4-fold increase in the relative ratio of free C-terminal *versus* Nd was observed for C2773S compared with the ratio detected for C2754W. This also indicates that VWF-C2773S dimerizes less efficiently at the C-terminus than VWF-C2754W does.

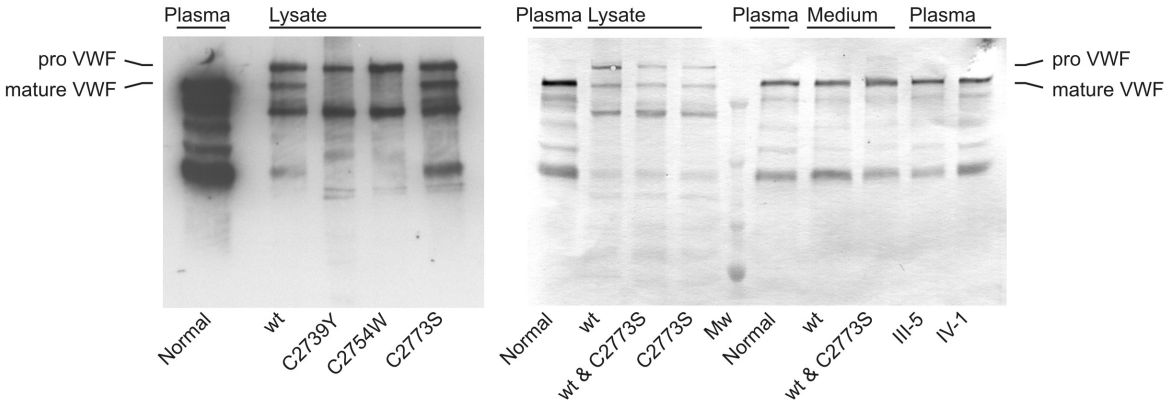

Fig. 5, SDS-PAGE analysis of the subunit size of recombinant VWF. Recombinant VWF in lysates or medium was reduced and separated by electrophoresis on denaturing 5% PAGE gels. VWF was detected with polyclonal antibody A082. Recombinant VWF from lysates of single transfections of wt and mutants; C2739Y, C2754W, and C2773S (**left panel**). Lysate and medium of recombinant VWF of wt, co-transfected (wt & C2773S) and mutant C2773S (C2773S), and for comparison reduced plasma VWF from patients III-5 and IV-1 (**right panel**). There is no difference in subunit size between the different recombinant C-terminal mutants and the pooled normal plasma (NP) and patient plasmas. The mutants C2739Y and C2754W show only proVWF and no mature VWF in lysates indicating reduced intracellular processing. Mw, molecular marker, top band corresponds to 250 kD.

Analysis of altered electrophoretic mobility of VWF

Analysis of reduced samples of VWF on denatured polyacrylamide gels (Fig. 5) excluded differences in the subunit size of wt and mutant VWF as the cause of the altered electrophoretic mobility observed for the dimeric VWF molecules (Fig. 3). In lysates of cells transfected with wt or mutant constructs, proVWF of all mutant constructs appeared to have the same mobility (Fig. 5, left panel). Also the apparent size of the mature VWF subunit of wt, C2773S and plasma VWF was similar. Further, no difference in subunit size was found between the mature VWF in plasma of patients III-5 and IV-1 and the VWF secreted by 293T cells co-transfected with wt and C2773S VWF constructs (Fig. 5, right panel). This suggests that the altered electrophoretic mobility of dimeric VWF is most likely explained by a difference in the conformation of the VWF-wt homodimers formed through conventional C-terminal pairing *versus* the conformation of homodimeric VWF-C2773S formed by dimerization at the N-terminus. Interestingly, while the

same intensity of proVWF and mature VWF was detected for rVWF-wt and rVWF-C2773S in cell lysates (Fig. 5, right panel), only the proVWF form was detected for rVWF-C2739Y and rVWF-C2754W (Fig. 5, left panel). This indicates similar intracellular processing of rVWF-wt and rVWF-C2773S suggesting that the C2773S subunits can participate in multimerization in the Golgi and are processed to mature VWF. In contrast, the absence of mature rVWF-C2739Y or rVWF-C2754W subunits in the lysates suggests hampered processing and intracellular retention and rapid degradation of these mutant subunits.

Discussion

Cysteine residues play a crucial role in determining the three-dimensional structure of VWF. All cysteine residues in VWF participate in either inter- or intrachain disulfide bonds (4). Biosynthesis of VWF is complex and involves the formation of proVWF dimers in the ER through covalent interchain disulfide bonds located in the CK domain of VWF (3). HMW multimers are generated in the Golgi apparatus through the formation of cysteine bonds at the N-termini of proVWF dimers. Concomitant removal of the VWF-propeptide renders HMW multimers consisting of mature VWF subunits linked together via interchain disulfide bonds at the C- and N-terminus.

Alterations of cysteine residues 2771 (10) and 2773 (11) in the CK domain of VWF have been identified in type 2A(IID) VWD patients, while changes of cysteines 2739 (6), 2754 (7), 2804 (8) and 2806 (9) were described in type 3 VWD. Although these mutations probably all cause a dimerization defect, as was demonstrated for 2771 (10), 2773 (11) and 2754 (7), they result in completely different VWD phenotypes. Interestingly, when we categorized the mutated cysteines by their involvement in either interchain or intrachain disulfide bonds, we noticed that the cysteines mutated in the two previously reported type 2A(IID) VWD patients might form interchain disulfide bonds and that the four cysteines mutated in type 3 VWD patients were all involved in the formation of intrachain disulfide bonds (3). Therefore, we hypothesized that the phenotypic discrepancies (VWD type 2A(IID) *vs* type 3) observed in patients with cysteine mutations in the CK domain may depend on whether the mutated cysteine residue participates in an inter- or intrachain disulfide bond.

First, we screened for mutations in the CK domain in an additional family with type 2A(IID) VWD anticipating, based on our hypothesis, to find a mutated cysteine that is involved in an interchain disulfide bond. We identified a transversion of guanine at nucleotide 8318 to a cytosine, resulting in the change of cysteine 2773 to a serine (C2773S). This cysteine is indeed, as expected, involved in an interchain disulfide bond (3). The C2773S mutation involves the same cysteine as the C2773R mutation previously reported by Schneppenheim *et al.* (11). Co-expression experiments of recombinant wt and mutant VWF exactly reproduced the type 2A(IID) phenotype of the heterozygous patients carrying this mutation: normal to high VWF:Ag level, absence of HMW multimers, and intervening bands between the normal multimers (Figs. 1 and 3).

To test the hypothesis that loss of inter- and intrachain disulfide bonds in the CK domain of VWF may give rise to different types of VWD, we compared the VWF phenotype of the novel C2773S mutation in 293T cells with that of two type 3 VWD mutations, C2739Y and C2754W that affect the formation of intrachain bonds. Whereas high production and secretion of rVWF-C2773S was demonstrated, the secretion of rVWF-C2739Y and rVWF-C2754W was strongly reduced (Fig. 2). In the conditioned media of cells co-transfected with wt and C2773S constructs as well as in the plasma of heterozygous carriers of the C2773S mutation, we observed a difference in the electrophoretic mobility of presumed homodimers of VWF-C2773S compared with that of homodimeric VWF-wt (Figs. 1-3), which was not observed for VWF-C2739Y and VWF-C2754W. The observed difference in electrophoretic mobility was not due to differences in subunit size of the reduced mutant, wt and plasma VWF (Fig. 5). Most likely it reflects a conformational change associated with the formation of N-terminally linked dimers by the VWF-C2773S subunit.

All three cysteine mutations that we have studied have impaired C-terminal dimerization as evidenced by excess of VWF monomer observed in media and lysates (Fig. 2) and the disrupted multimerization (Figs. 2 and 3). Furthermore, the C2773S and C2754W mutant proteins display VWF dimers despite the C-terminal dimerization defect (Figs. 2-3 and 4B). Utilizing ADAMTS13 proteolysis of VWF in combination with SDS-PAGE and immunoblotting with polyclonal and monoclonal antibodies, we could demonstrate that these mutant VWF dimers are the result of N-terminal dimerization (Fig. 4). The dimerization defect at the C-terminus of VWF-C2773S is nearly complete showing mainly the free C-terminal fragment (1C)

and N-terminally formed VWF dimers (Nd) and almost no C-terminally linked VWF dimers (Cd) (Fig. 4). The majority of the VWF-C2773S dimers are thus formed through interchain disulfide bonds at the N-terminus. The detection of low concentrations of tetrameric VWF-C2773S in the media of single transfected cells (Fig. 2) suggests the participation of more than one cysteine residue in dimerization in the CK domain of VWF which supports the model proposed by Katsumi *et al.* (3) where three cysteine residues (C2771, C2773 and C2811) sustain dimerization rather than C2773 alone. The mutation of cysteine 2773 has previously been shown to have a slightly greater impact on dimerization than mutation of cysteine 2771. While recombinant expression of a full-length VWF construct encoding VWF-C2773R resulted in the secretion of tetramers (7), expression of VWF-C2771S and VWF-C2771Y led to the secretion of octamers (10). In fact, when C2811 was mutated to an alanine in a C-terminal VWF construct (aa 2720-2813), recombinant dimers of VWF were still secreted while altering either C2771 or C2773 to an alanine prevented VWF from dimerization (3). These data indicate that the cysteines at position 2771 and 2773 play a key role in the dimerization of VWF and that the cysteine positioned at 2811 most likely only has a minor contribution to normal dimerization of VWF.

Recombinant VWF-C2754W also forms N-terminal dimers, but based on the ratio of dimers formed at the C- and N-terminus (Cd and Nd) the ability of C2754W to dimerize at the C-terminus seems less compromised than for VWF-C2773S. This may be explained by the fact that C2754W is not directly involved in the dimerization through interchain disulfide bonds (3), but probably hampers dimerization through conformational changes of the VWF monomer. The conclusion that rVWF-C2754W has more residual C-terminal dimerization capacity than rVWF-C2773S is also supported by the fact that single transfections of C2754W show a considerable amount of trimers. The other type 3 VWD mutation C2739Y also shows trimers in cell lysates of single transfections (Fig. 2). The formation of trimers requires N- as well as C-terminal disulfide bond formation. However, rVWF-C2773S does not show trimers in single transfection experiments indicating a strong preferential formation of N-terminal disulfide bonds. The formation of tetramers and no trimers for rVWF-C2773S (Fig. 2) may be explained by the abundance of monomeric rVWF-C2773S in comparison to the minute amounts of dimeric rVWF-C2773S in the ER. In the Golgi rVWF-C2773S dimers are outnumbered by monomeric rVWF-C2773S leading to the addition of

C2773S monomers by multimerization which results in the generation of tetramers.

Based on the different characteristics of mutated cysteines in the CK domain involved in either type 2A(IID) or type 3 VWD, we propose the following model to explain the differences in phenotype. So far, the mutated cysteines in the CK domain identified in type 2A(IID) VWD are all involved in the formation of interchain disulfide bonds while all the cysteines mutated in type 3 VWD are involved in forming intrachain disulfide bonds. Disruption of an intrachain disulfide bond severely affects the conformation of the VWF monomer. Due to this conformational change, the mutant VWF is retained in the cell and probably cannot efficiently dimerize in the ER and pass through to the Golgi. The abnormal intracellular processing is illustrated by our finding of only proVWF and not mature VWF in lysates of transfections with C2739Y and C2754W (Fig. 5). This intracellular retention results in a quantitative reduction of VWF, mimicking a null allele. In the homozygous situation this leads to nearly complete absence of VWF. In the heterozygous state this leads to a reduction of about 50% of VWF as most of the mutant subunits will be retained in the ER, but the normal VWF dimers (from the normal allele) will be transported to the Golgi and form normal multimers. The small amount of mutant monomers that do pass to the Golgi can contribute to the formation of odd-numbered multimers by N-terminal pairing as we have shown before (12). In heterozygous carriers or co-transfections of wt with mutant C2739Y or C2754W constructs, the minute amount of mutant subunit that passes the ER to participate in multimerization in the Golgi would be outnumbered by the large majority of wt subunits. Consequently, this would not lead to a dominant negative effect (12). In this model, loss of cysteines in the CK domain that are involved in intrachain bonds will lead to a recessive, quantitative VWF deficiency, *i.e.*, type 3 VWD.

On the contrary, alterations of cysteines involved in interchain bonds are not expected to have a severe effect on the secondary structure of the VWF monomer. Although loss of these cysteines results in a dramatic decrease in the ability to form C-terminal dimers, these mutant subunits can pass from the ER to the Golgi. VWF dimers may then be formed through dimerization at the N-terminus (Fig. 4). Co-expression of wt and mutant alleles (as in heterozygous patients) would result in a mixture of normal dimerization at the C-terminus of wt VWF and addition of the mutant monomers to the wt dimers through N-terminal pairing (Figs. 1

and 3). This leads to the characteristic type 2A(IID) multimer pattern in which further multimerization is halted by the addition of monomeric mutant subunits to the N-terminus of the growing multimer. This leads to a dominant negative effect on the multimerization as the number of mutant monomers transported to the Golgi is relatively high. The effect is progressive with increasing amounts of mutant in co-transfections as illustrated in Fig. 3. In this model loss of cysteines in the CK domain involved in formation of interchain bonds will lead to a dominant negative defect of VWF with abnormal multimers, *i.e.*, type 2A(IID) VWD.

In conclusion, we have identified a novel mutation, C2773S, that causes type 2A(IID) VWD. Furthermore, we have shown that mutations causing a dimerization defect at the C-terminus of VWF can form dimers through N-terminal pairing of VWF monomers. Based on quantitative and qualitative characteristics of mutated cysteines associated with type 2A(IID) and type 3 VWD, we propose that mutated cysteines in the CK domain of VWF will lead to a recessive, quantitative VWF deficiency if intrachain disulfide bonds are involved, and to a dominant negative, qualitative defect of VWF if interchain bonds are involved.

Acknowledgements

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Evaluation of the von Willebrand factor Y1584C polymorphism as a potential risk factor for bleeding in patients receiving anticoagulant treatment with vitamin K antagonists

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Introduction

The number of patients receiving anticoagulant treatment with vitamin K antagonists (VKA) is still increasing and a substantial number of these patients experience major bleeding complications as a consequence of this therapy, 1-3% yearly (1,2). It is of utmost importance to prevent bleeding complications from anticoagulant treatment. Identification of risk factors for bleeding complications will help to improve the safety of VKA treatment.

It is well known that the intensity of VKA treatment is the major determinant of the risk of bleeding in VKA-treated patients. However, other factors also contribute to the risk and play a significant role (1,3). In the search for novel risk factors associated with bleeding in VKA treated patients, the FACTORS (FACTors in ORal anticoagulation Safety) study was initiated (3). The FACTORS study is a case-control study including patients on VKA treatment that have (cases) or have not (controls) experienced major bleeding. The definition of major bleeding was: bleeding leading to death, hospitalization or a decrease of more than 1.25 mM of hemoglobin; intracranial bleeding; and muscle, joint, or intraocular bleeding. The indications for VKA treatment of the patients participating in the FACTORS study were atrial fibrillation, venous thromboembolism, post operative prophylaxis, mechanical heart valve, vascular surgery, and ischemic heart disease. In the study, cases and controls were recruited from the Thrombosis Services in Leiden and Amsterdam and they were matched for the indication of VKA treatment, age, gender, intensity and type of VKA (acenocoumarol or phenprocoumon). The FACTORS study was previously described in detail (3). It has been shown that high levels of soluble thrombomodulin (s-TM) are associated with an increased risk of bleeding (4). However, the risk was only linked to the highest quartile of s-TM in the FACTORS study (3). Thus additional, possibly novel genetic risk factors may play a role in the bleeding in patients treated with VKA.

A candidate genetic risk factor is the von Willebrand factor (VWF) Y1584C polymorphism. This polymorphism was shown to be linked to increased susceptibility of VWF to proteolysis by ADAMTS13 (5). The VWF Y1584C polymorphism was found at a frequency of 1% (heterozygotes) in two different cohorts of normal individuals (2/200 (5) and 1/100 (6)). A much higher frequency of 14% of this polymorphism was found in type 1 von Willebrand disease (VWD)

patients in a Canadian study (6). However, the polymorphism did not always co-segregate with VWD and some of the heterozygous carriers were unaffected and showed normal VWF antigen (VWF:Ag) levels and VWF multimer patterns (5,6). Even in the presence of normal VWF:Ag levels this polymorphism may affect the formation of the platelet plug through increased *in vivo* proteolysis of VWF multimers under shear stress as suggested by Bowen *et al.* (5). Hence, the effect of this polymorphism on VWF multimer survival *in vivo* and primary hemostasis may be substantial. Consequently it may modulate the risk for bleeding and may be considered a potential general risk factor for bleeding, particularly in patients already hemostatically challenged by VKA treatment.

Materials and methods

To investigate whether VWF Y1584C is indeed associated with bleeding, we screened 327 patients from the FACTORS study for the presence of the VWF Y1584C polymorphism; 110 of these were patients with bleeding episodes during VKA treatment (cases) and 217 were patients without bleeding episodes during VKA treatment (controls).

The screening was performed on genomic DNA by amplification of exon 28 by PCR with a forward oligonucleotide specific for the VWF gene, 5'-TGG TTCTGGATGTGGCGTTC (complementary to nts 24/1015-1034), and a reverse oligonucleotide, 5'-CAAGGCCATGCCAGCCCTCG (complementary to nts 24/ 1622-1641, numbering according to Mancuso (7)). The use of a gene-specific oligonucleotide prevents inadvertent co-amplification of the VWF pseudogene. Subsequently, the 627 bp PCR product was digested with the restriction enzyme *Acc*65I. Homozygosity for the common allele would yield two fragments of 269 and 358 bp, whereas heterozygosity would yield three fragments of 269, 358 and 627 bp, due to the loss of the *Acc*65I site in the 1584C allele.

Results and discussion

In the population of 327 individuals, two patients that were included because of bleeding (cases) were heterozygous for the polymorphism (Table I). The first patient was an 81-year old male using VKA for atrial fibrillation and who had experienced gastrointestinal bleeding. His VWF:Ag level was 196%. The second

Table I: Distribution of the VWF Y1584C polymorphism in cases and controls

VWF	Case Bleeding +	Control Bleeding –	OR*	CI 95%‡
YY1584	108	217	1†	
YC1584	2	0	∞	0.37- ∞

*Odds ratio. †Reference category. ‡According to (8).

patient was a 68-year old female who received VKA for atrial fibrillation and ischemic stroke. She experienced muscle hemorrhage and her VWF:Ag level was 155%. No heterozygotes were observed in the control group of 217 individuals that did not experience major bleeding.

In conclusion, we present some evidence that the VWF Y1584C polymorphism may be associated with bleeding in VKA-treated patients. However, because of the rarity of this polymorphism in our population, a much larger group of patients and controls would be required to assess reliably the bleeding risk associated with this polymorphism. In the two patients tested positive for the Y1584C polymorphism VWF levels were high. Although the polymorphism was previously found to be associated with type 1 VWD (5), it can not be considered a causative VWD mutation. The contribution to bleeding risk in other patient categories than those treated with VKA remains to be determined.

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General discussion

Introduction

Since the publication in 1926 by Erik von Willebrand on "hereditary pseudohemophilia" the lack of functional von Willebrand factor (VWF) has been identified as the cause of this bleeding disorder (1-3). This factor has been shown to play an important role in both primary and secondary hemostasis. In primary hemostasis, it functions as a molecular glue between platelets and subendothelial structures (reviewed in (4,5)); in secondary hemostasis it shields FVIII from premature activation and inactivation by proteases and from receptor-mediated clearance (6-8).

Von Willebrand disease (VWD) is the most common bleeding disorder in the population. It is characterized by symptoms ranging from very mild to severe bleeding, and is caused by alterations in the very large VWF gene. These may influence the level of VWF (quantitative defect; type 1 or type 3) or may affect the function of VWF (qualitative defect; type 2A, 2B, 2M, 2N) (9). To yield functional VWF, correct processing is paramount. Especially important is the formation of disulfide linked bonds. Intrachain linking is essential for the monomer structure. Interchain linking between the CK domains of two monomers is required for dimerization and interchain linking across D3 domains of proVWF dimers is necessary for multimerization. All cysteine residues in the mature VWF subunit (169 or 8.2%) participate in these intra- or interchain disulfide bonds (10). The interaction between proVWF dimers ultimately yields high molecular weight VWF that is active in primary hemostasis in the bloodstream.

The main aims of the studies reported in this thesis were to examine how loss of cysteines located in different domains of VWF results in quantitative and qualitative VWF defects; how these mutations interfere with dimerization and multimerization; and how they influence intracellular routing, secretion and clearance of VWF. The effects of mutated cysteines in different domains of VWF are reported in **Chapters 2-5**. Finally, in **Chapter 6** we evaluated whether the additional cysteine in carriers of the Y1584C mutation in VWF is associated with additional risk of bleeding in patients treated with vitamin K antagonists.

Cysteine mutations in the CK domain

The 151 C-terminal amino acids in VWF are sufficient to support dimerization of proVWF molecules in the endoplasmic reticulum (ER) (11). The 90 most C-terminal amino acids comprise the cystine knot (CK) domain (12). This is a motif that is common in proteins that form dimers and oligomers. In VWF this motif consists of 11 cysteine residues of which eight are involved in intrachain disulfide bonds forming a ring-like structure and of which the remaining three are suggested to form interchain disulfide bonds supporting dimerization.

We were intrigued by the fact that replacement of cysteine residues in the CK domain causes two completely different VWD phenotypes. Loss of cysteines C2739 (13), C2754 (14), C2804 (15), and C2806 (16) results in type 3 VWD with nearly complete absence of VWF and loss of C2771 (17) and C2773 (18) results in type 2A(IID) VWD with a structural defect of VWF (Fig. 1). Interestingly, we noticed that cysteines described in type 3 VWD are all involved in intrachain disulfide bonds, whereas those in type 2A(IID) VWD are proposed to be involved in formation of interchain disulfide bonds (12). How missense mutations result in a quantitative defect is an especially intriguing aspect, since point mutations are not expected to either affect the stability of mRNA or translation and thus do not provide a straightforward mechanism for a quantitative defect.

We therefore hypothesized that loss of intrachain disulfide bonds may result in a conformational change leading to a quantitative VWF defect when the mutant monomer is retained in the ER and subsequently degraded. This would mimic the phenotype of a null allele. We further hypothesized that loss of an interchain disulfide bond has less impact on the monomer structure of VWF allowing both mutant and normal VWF to enter the Golgi and participate in random multimerization. This would explain the dominant negative effect that these mutations have on multimerization of VWF and the occurrence of odd-numbered VWF multimers in the plasma of heterozygous type 2A(IID) patients. Furthermore, it would explain the strictly qualitative effect on VWF as only a minor amount of VWF would be retained in the cell.

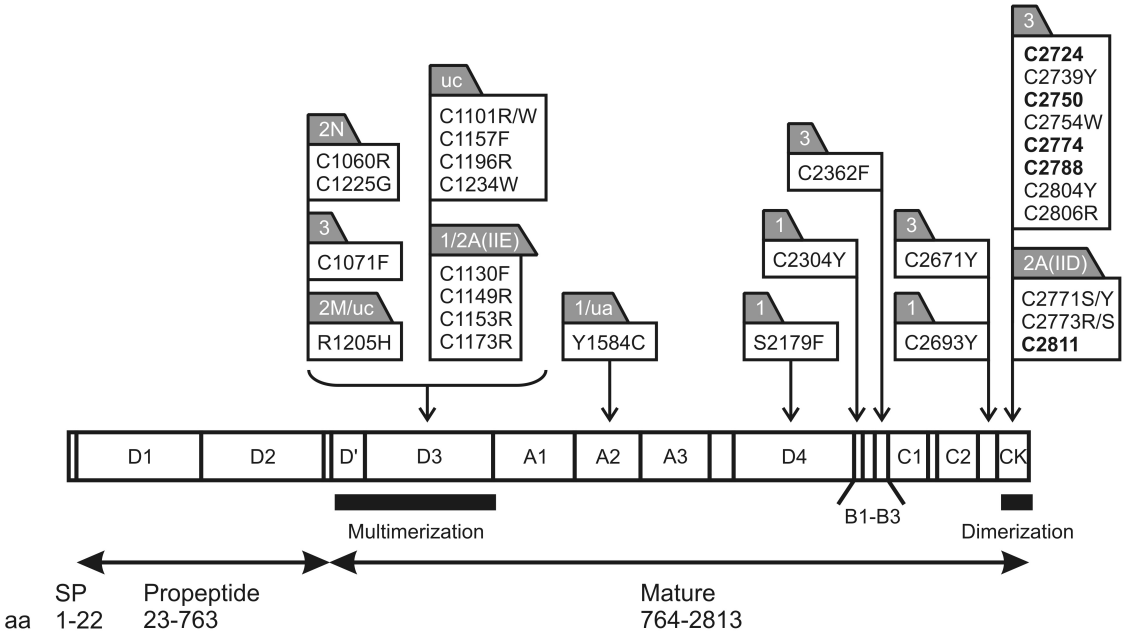

Fig. 1, localization in VWF of selected residues according to VWD phenotype. In this schematic overview of the VWF preproprotein, mutations which are located in the same domain and cause the same VWD phenotype (grey tabs) are grouped. Mutations are discussed in different sections of the text. Cysteines in the CK domain, for which no mutations have yet been found (bold), are arranged according to the predicted VWD phenotype, based on their role in the formation of intra- or interchain disulfide bonds. Signal peptide (SP), VWF-propeptide and mature VWF are indicated as well as the domains (A-D, CK) and regions involved in dimerization (CK domain; aa 2724-2813) and multimerization (D'-D3 domains; aa 769-1242). Letters and numbers in grey tabs indicate different VWD phenotypes. ua, unaffected individuals; uc, unclassified VWD.

Quantitative and qualitative VWF defects: Intrachain versus *interchain disulfide bonds*

Type 3 VWD

Expression studies by us (**Chapter 2a**) and by Schneppenheim and colleagues showed that missense mutations of cysteines could reproduce the quantitative VWF defect observed in type 3 VWD and thereby mimic the effect of a null allele (14,19). The quantitative defect appeared to be caused by the strong intracellular retention of the mutant protein (19), which is supported by the presence of only minute amounts of VWF in platelets (14). We observed that for mutant C2739Y and C2754W VWF only unprocessed proVWF was present intracellularly, while for the wt mature VWF as well as proVWF was observed (**Chapter 5**). This confirmed

that loss of these intrachain bonds indeed severely affected the processing of proVWF subunits by preventing transport to the Golgi apparatus where removal of the VWF-propeptide occurs. The strong effect that loss of these disulfide bonds has on processing may be explained by their participation in the formation of the cystine knot in which intrachain disulfide bonds participate in a ring-like structure (12). The loss of this structure probably severely affects the monomer conformation resulting in a misfolded protein that is retained in the ER.

Katsumi and co-workers identified the following intrachain disulfide bonds: C2724-C2774, C2750-C2804, C2754-C2806 and C2739-C2788 (12). Apart from the C2739Y and C2754W mutations (13,14,19), mutations of C2804 (15) and C2806 (16) have also been implicated in a type 3 VWD phenotype (Fig. 1). This further supports our hypothesis that intrachain disulfide bonds severely affect the VWF monomer structure since loss of either C2754 or C2806, which together form one intrachain disulfide bond, results in the same severe quantitative VWD phenotype. Additional evidence for our hypothesis is found in co-transfections of wt and mutant VWF (C2739Y or C2754W) leading to ~50% reduction of secreted VWF:Ag also mimicking the effects of a null allele (19). Mutations of cysteines C2724, C2750, C2774, or C2788 have not yet been reported. Our hypothesis, however, predicts that mutations at these positions all will result in a quantitative defect as seen in type 3 VWD.

Type 2A(IID) VWD

Not all cysteine mutations in the CK domain result in a quantitative VWF defect. Some result in a rare qualitative VWF defect, called type 2A(IID) VWD. Changes of C2771 to tyrosine or serine (17) and C2773 to arginine (18) were detected in this uncommon VWD phenotype (Fig. 1). The importance of both C2771 and C2773 in dimerization of VWF was stressed by the complete abolishment of the formation of VWF dimers due to the incorporation of either C2771A (12) or C2773A/R (12,18) in C-terminal fragments of VWF. In **Chapter 5** we screened a family with type 2A(IID) VWD anticipating to identify a mutated cysteine involved in an interchain disulfide bond. Indeed, in two affected individuals we identified a novel mutation of C2773S.

In agreement with our hypothesis the introduction of the C2773S mutation in VWF did not lead to impaired expression or secretion of VWF (Chapter 5). In contrast, even higher extracellular levels of C2773S VWF were observed than for

wt VWF. Similar results were observed in expression studies of C2773R VWF (14) and C2771Y/S VWF (17). Furthermore, the observation of both mature and proVWF C2773S in the intracellular compartment supports the idea that loss of interchain disulfide bonds only has a minor effect on conformation and the initial processing of proVWF (Chapter 5). Finally, all these mutations have a major effect on dimerization of VWF and subsequently on multimerization. This ultimately results in secretion of mainly monomers, dimers, tetramers, some trimers, and sometimes hexamers and octamers of VWF (Chapter 5) (14,17).

In co-expression studies we and others were able to recreate the dominant 2A(IID) VWD phenotype with its normal to high levels of VWF:Ag and peculiar VWF multimer pattern (Chapter 5) (17,20). This pattern is characterized by odd-numbered multimers located in-between the normal even-numbered multimers and loss of intermediate- and high molecular weight multimers (21). Together these data support our hypothesis that loss of interchain disulfide bonds in the CK domain only induces minor changes of the monomer conformation which results in a qualitative VWF defect in the heterozygous state. This is caused by the incorporation of the mutant monomer in the growing multimer in the Golgi apparatus, thus hampering further multimerization of VWF.

Additionally, it may be interesting to investigate whether replacement of proline residues have a strong effect on the structure and conformation of the CK domain of VWF. This may result in a similar phenotype as loss of cysteine residues. To date only two such mutations have been reported in the VWF database (http://www.sheffield.ac.uk/vwf). These are the alteration of P2776 and P2781 to lysine and serine, respectively. They result in two separate phenotypes, the former leads to a quantitative VWF defect (type 1 VWD) while the latter results in a qualitative defect (type 2M VWD). The absence of additional data, expression studies and a three-dimensional structure of this region makes it hard to generalize the effects of such mutations on VWD phenotype.

In summary, loss of intrachain bonds in the CK domain of VWF has been found to result in type 3 VWD in homozygous patients, whereas loss of interchain disulfide bonds results in type 2A(IID) VWD in heterozygous patients. Investigation of other cysteine residues in the CK domain may provide more clues as to whether the model that links loss of intrachain bonds to type 3 VWD and loss of interchain bonds to type 2A(IID) VWD may be generalized.

N- and C-terminal dimerization of VWF

The observation of tetramers (C2773R/S) and even hexamers and octamers (C2771Y/S) of full-length VWF (**Chapter 5**) (14,17) indicated that although alteration of either C2771 or C2773 in VWF has a profound effect on dimerization of C-terminal VWF fragments (12,18) this effect is milder in full-length VWF. The difference in the effect of these mutations on dimerization of VWF between truncated (12,18) and full-length VWF (Chapter 5) (14,17) may possibly be due to additional interactions between two proVWF molecules in full-length VWF. These interactions may have a stabilizing effect on the conformation of the full-length protein and thereby facilitate the formation of disulfide bonds in the CK domain. This may not occur in truncated VWF and therefore result in stronger effects of the introduced mutations on dimerization of the truncated VWF protein than in the full-length version.

The identification of VWF multimers higher than dimers of full-length C2771 or C2773 VWF shows that C-terminal dimerization is still possible to some degree in full-length C2771Y/S (17) and C2773R/S VWF(Chapter 5) (14). This suggests either that two C2771 or two C2773 residues pair, or that C2771 pairs with C2773, in which case a third disulfide bond is expected (see below) and is necessary to explain the observed oligomers in the mutants. If dimerization would be solely dependent on either C2771 or C2773, the highest molecular weight species would be VWF dimers assuming that N-terminal pairing of mutant monomers will occur in the Golgi apparatus. N-terminal pairing was previously suggested by Schneppenheim *et al.* and Enayat *et al.* (14,17). It was, however, not directly demonstrated that C2771Y, C2771S or C2773R form N-terminal dimers.

In **Chapter 5** we addressed this issue by treatment of recombinant C2773S VWF with ADAMTS13. This will allow the separation of N-terminal and C-terminal fragments, which subsequently can be detected with specific monoclonal antibodies. As anticipated, N-terminal dimers and free C-terminal chains were readily detected, whereas C-terminal dimers of C2773S VWF were barely visible. This clearly shows that mutant C2773 is able to form dimers through N-terminal pairing in the Golgi and that the mutant subunit only has low residual binding capacity at the C-terminus, which explains the occasional formation of tetramers. In co-transfections of C2773S and wt, a mixed pattern of both N- and C-terminal dimers and free C-terminal chains was observed, indicating normal dimerization of wt as well as N-terminal pairing of mutant subunits. The observation of

odd-numbered multimers in the heterozygous transfection indicates that mutant subunits also interact with the wt multimers in the Golgi apparatus, thus explaining the dominant negative effect on multimerization.

CK structure

Katsumi and co-workers suggested that neighbouring proVWF molecules are linked by either one or three interchain disulfide bonds involving residues C2771, C2773 and C2811 (12). They showed that residues C2771 and C2773 are crucial for dimerization of a CK VWF fragment while C2811 was not. Therefore, a model is favoured in which three cysteines rather than a single one form the interchain disulfide link needed for dimerization (12). How exactly these interchain disulfide bonds link these subunits is unknown. The model based on the crystal structure of transforming growth factor β2 (TGF-β2) which has a similar CK structure suggests crossed linkage between C2771 and C2773 of adjacent subunits and a C2811-C2811 interchain bond (12). Even though the absolute requirement of C2771 and C2773 and the minor effect of changes of C2811 are in line with this model, it does not explain the observation of up to octamers in homozygous transfections of C2771Y or C2771S (17) whereas only up to tetramers is observed in homozygous transfections of C2773R or C2773S (**Chapter 5**) (14). If crosslinkage occurs between C2771 and C2773 in two proVWF subunits, then the effect of C2771S and C2773S on dimerization and multimerization of VWF is expected to be similar, since in each case both bonds are lost. A comparative study utilizing the same expression system and multimer analysis of VWF still has to show whether this apparent milder effect of C2771Y/S on multimerization is biologically relevant or a result of the expression system. It is interesting to speculate whether the apparent milder effect of C2771Y/S is related to how C2771, C2773 and C2811 are linked.

It may be that the structure of the VWF CK domain deviates more from the structure of TGF-β2, since the VWF CK domain lacks one of the TGF-β2 cysteines and contains four additional cysteines residues (12). In light of these differences, one may envisage that the three interchain disulfide bonds are formed between the corresponding cysteines of the two subunits, *e.g.*, C2771-C2771, as opposed to the crossing-bonds model. If a C2771 or C2811 interchain bond is lost in this hypothetical model, this may have a smaller effect on the structure, still allowing dimerization to occur, while loss of the C2773 bond located in between these

bonds may have a stronger effect on the conformation of the protein. The three-dimensional structure of the CK domain is probably the only way to resolve this issue, since distinct separation of intrachain and interchain disulfide bonds using proteolytic fragments of VWF dimers is extremely difficult.

Cysteine mutations in the D3 domain of VWF

Several missense mutations of cysteine residues in the D3 domain that is required for multimerization (22) have been reported in the VWF mutation database (http://www.sheffield.ac.uk/vwf). Interestingly, these mutations lead to different phenotypes. Some cause quantitative defects: C1130F and C1149R (type 1 VWD, (23)) and C1071F (type 3 VWD, (24)); others cause qualitative defects: C1060R (type 2N VWD, (25)), C1153Y and C1173R (type 2A (IIE), (26,27)); C1101R/W, C1157F, C1196R and C1234W cause as yet unclassified VWD phenotypes (http://www.sheffield.ac.uk/vwf, unpublished data) (Fig. 1).

Although type 1 VWD patients represent the largest group of VWD cases, only few mutations had been reported until recently. This has changed since the start of the European multicentre study "Molecular and Clinical Markers for Diagnosis and Management of Type 1 VWD" (MCMDM-1VWD) which aimed to characterize the genotype-phenotype relationship in type 1 VWD. The study led to the identification of 126 candidate mutations throughout the VWF gene (28). The majority of these (81%) are missense mutations and of these 29% are found in the D' and D3 domains. Some amino acids have a higher mutation frequency than others. Interestingly, one of those is C1130, which was altered to three different amino acids, namely glycine, arginine and phenylalanine (28). The clinical penetrance of the C1130G/R/F mutations was very high in the investigated families (93%) (29). The patients were characterized by very low VWF:Ag levels (MCMDM-1VWD, personal communication). This is in line with the dominant effect of mutations described in type 1 VWD patients (23,29). A similar phenotype has been described for C1149R (23). Our hypothesis that the strikingly low VWF:Ag level in patients with these mutations is caused by an interaction of mutant, C1130F or C1149R, and wt VWF subunits resulting in intracellular retention, was only partly supported in expression studies of these proteins (**Chapter 2a**) (19). Our results and those of the MCMDM-1VWD study showed normal synthesis and decreased secretion of the mutant protein (19,30). Most likely two factors

contributed to the decreased secretion of VWF. Firstly, intracellular retention of C1130F or C1149R homodimers, and heterodimers of wt and these mutant proteins (19,23,31), and secondly, proteasomal degradation of retained wt and C1149R heterodimers (31). Although loss of C1130 and C1149 do affect secretion and retention of VWF, the moderate effects in *in vitro* culture cannot explain the strikingly low levels of VWF in heterozygous patients. This suggests that other factors like *in vivo* clearance may contribute to the type 1 VWD phenotype (**Chapter 3**) (32).

All mutations, C1130G/R/F and C1149R, are associated with the absence of HMW multimers in plasma (19,30). In co-transfections of wt and C1130F or C1149R VWF, the observed small reduction in the HMW multimers seems to be in accordance with the plasma multimer pattern in patients (19,23). Multimerization of VWF is facilitated by the VWF-propeptide and occurs under slightly acidic conditions (pH<6.5) in the Golgi apparatus (4,33). In **Chapter 3** the interaction of C1130F and C1149R VWF with the VWF-propeptide was studied at different pH (32). The interaction was severely reduced at all pHs (5.2-7.4) which may explain the observed multimerization defect *in vitro* as well as *in vivo* (32). The effects of the C1130F and C1149R mutations may stretch even further considering the crucial roles of the D'-D3 domain of VWF in the interactions with the VWF-propeptide and P-selectin. The former interaction facilitates storage of VWF to Weibel-Palade bodies (WPB) (33); through the latter, P-selectin is recruited to WPBs (34). P-selectin may have a key role in *in vivo* cleavage by anchoring released ultra-large VWF multimers and thereby facilitating their unfolding and subsequent cleavage by ADAMTS13 (35). Finally, the physiological function of VWF was suggested to depend on its tubular storage in endothelial WPBs (36). It was proposed that these tubules are VWF multimers coiled into a constrained spring at low pH which requires a pH-dependent interaction of the VWF-propeptide with VWF, and that, upon exocytosis, dissociation of the VWF-propeptide-VWF complex allows unfurling of VWF tubules to long platelet-catching VWF filaments. This new mechanism could explain the rapid exocytosis of ultra-large VWF without generating tangled VWF filaments.

Since obstruction of the above described interactions would probably affect the function of VWF in the bloodstream, it would be interesting to further investigate the C1130F, C1149R and other missense mutations in the D'-D3 domains of VWF. These mutations may decrease the interaction between VWF

and the VWF-propeptide, which may affect storage of VWF in WPBs, resulting in tangled VWF instead of organized VWF tubules (36). Upon exocytosis, tangled VWF may be less capable of recruiting platelets (36). However, if the mutations do not interfere with the tubular storage of VWF, but instead affect its binding to P-selectin then ultra-large VWF may be secreted, potentially with thrombogenic effect (34,35). Alternatively, mutations leading to increased affinity of VWF to P-selectin may result in rapid cleavage of ultra-large VWF to LMW species (35,37), which are less efficient in recruiting platelets (36) and may have a negative effect on the ability of VWF to support primary hemostasis.

Another important problem that has to be tackled is the troublesome discrimination between the diagnosis of type 1 or type 2 VWD of these patients, which often depends on the generation of a reliable and reproducible multimer pattern. Standardization of multimer analysis by the European Study of type 1 VWD has facilitated this discrimination and showed that mutations C1130G/R are associated with subtle multimerization defects in patients previously diagnosed with type 1 VWD (30). Although the quantitative defect is the most prominent phenotype in these patients, the multimer patterns of the index cases were abnormal with a relative decrease or even absolute loss of HMW multimers which points to a qualitative type 2 (IIE) VWF defect (MCMDM-1VWD, personal communication). Incorrect diagnosis may result in an overestimation of the type 1 VWD patient group at the expense of the type 2 VWD group and more importantly it may affect the choice of treatment for these patients.

Interestingly, the MCMDM-1VWD study also suggested that the C1130F/R mutations may not only affect quantity and quality of VWF multimers, but may also have an effect on VWF:FVIII binding activity (VWF:FVIIIB) (38). This is in line with our findings of reduced VWF:FVIIIB of C1130F and C1149R in Chapter 3 (32). The FVIII binding site is located in the amino-terminal 272 aa residues of VWF (39). Other cysteine mutations in the D3 domain have been reported to have reduced or absent FVIII binding activity (40-42). C1060R (40), C1157F (42), C1225G (41) and C1234W (42) affect the affinity of VWF to bind FVIII to a varying degree suggesting that missense mutations located outside the FVIII binding site itself (39) change the affinity for FVIII, possibly due to conformational changes. The C1060R, C1157F and C1234W mutations all showed a strong effect on multimerization resulting in loss of HMW multimers, whereas C1225G only leads to a mild reduction of HMW multimers (40-42). C1225 is the only one of the above

cysteine residues that has been suggested to be involved in an interchain disulfide bond (43,44). This suggests that mutations of cysteines involved in the formation of interchain disulfide bonds do not affect the three-dimensional structure of the protein to the same extent as mutations of cysteines involved in intrachain disulfide bonds.

The number of interchain disulfide bonds in the D3 domain is uncertain since it is not clear for all cysteine residues in this domain whether they participate in an inter- or intrachain disulfide bond. This makes it highly speculative to predict the effects of these mutations on biosynthesis, multimerization or FVIII binding capacity to their possible involvement in inter- or intrachain bond formation. However, the effects of these mutations and the relation to their involvement in inter- or intrachain disulfide bonds remains an interesting question that may be answered by the resolution of the crystal structure of VWF or VWF domains which will reveal the true number and location of cysteine bonds linking the separate VWF subunits as well as the intricate web of intrachain disulfide bonds.

Cysteine mutations outside the CK and D3 domains

C2362F VWF

The C2362F mutation has been identified in several Italian families with an autosomal recessive severe quantitative VWF defect (45). It is located in the B3 domain of VWF to which so far no specific structural or functional property has been assigned (Fig. 1). Only one other cysteine mutation has been found in this region, C2304Y, which is located in the neighbouring B1 domain and leads to a partial quantitative defect of VWF (46).

The C2362F (**Chapter 4**) (47) as well as the C2304Y (46) mutation results in poor secretion and strong intracellular retention of VWF, which completely explains the quantitative VWF defect observed in patients homozygous for C2362F (47). This was unexpected since increased *in vivo* proteolysis has been suggested to contribute to the quantitative defect (48). Both mutations had similar effects on processing of VWF resulting in lack of HMW and IMW VWF multimers (46,47). Unfortunately, no data on co-expression of wt and C2304Y VWF have been published until now. This makes it difficult to identify a common effect of mutated cysteines in this region of VWF.

Our results suggest that the major contributor to the quantitative defect is the loss of the disulfide bridge in which C2362 normally participates (47). This is presumably an intrachain disulfide bond since this region is not involved in dimerization or multimerization of VWF. Loss of this covalent bond most likely induces a conformational change hampering processing of mutant proVWF to mature VWF, as suggested by the relative abundance of proVWF in cell lysates (47), and resulting in intracellular degradation of most of the mutant protein. This is supported by undetectable and barely detectable VWF:Ag in platelets of patients homozygous or compound heterozygous for the C2362F mutation, respectively, whereas it was readily detectable in platelets of a heterozygous subject (47). Support for a conformational change is obtained from the alteration in electrophoretic mobility of recombinant as well as the plasma C2362F VWF multimers, while no size difference between wt and mutant VWF subunits of reduced VWF was observed (47). Interestingly, such an increase in electrophoretic mobility of the mutant protein was also observed for C2304Y VWF in the MCMDM-1VWD study (46). A conformational change of the VWF mutant subunit may explain the quantitative defect through intracellular retention and degradation of VWF (47). The smeary VWF multimer pattern observed in the plasma of the homozygous patient may also be explained by the altered cysteine residues in VWF as has been reported previously (27).

We were unable to detect increased *in vivo* proteolysis of the mutant C2362F subunit by ADAMTS13 (47). However, a system utilizing a more physiological setting, *e.g.*, unfolding VWF by shear flow instead of urea, may be capable of detecting a subtle increase in susceptibility to this protease that is overlooked in the rather crude approach of chemical unfolding of VWF. Although the decreased half-life of endogenous C2362F VWF supports the suggestion of increased clearance via a receptor or proteolysis, further investigation of the contribution of these factors to the quantitative defect is probably of limited value since the level of secreted C2362F VWF is already very low (47). It is still puzzling how the very low VWF:Ag observed in these patients results in a sustained high level of FVIII:C even four hours after initiation of DDAVP infusion (47,49). This aspect remains especially intriguing, considering the normal binding affinity of C2362F VWF for FVIII:C in all patients (48).

C2671Y VWF

The C2671Y mutation (**Chapter 2a**) is located in the connecting region between the C2 and the CK domain of VWF (Fig. 1). It was found in a patient with type 3 VWD who was compound heterozygous for C2671Y and a complete deletion of the other allele (50). In this region only one other cysteine mutation, C2693Y, has been identified in a patient with type 1 VWD in the MCMDM-1VWD study (personal communication). Surprisingly, expression studies of C2671Y VWF did not at all reproduce the severe quantitative defect that was observed in the patient (19). Co-expression of wt and C2671Y VWF generated a normal multimer pattern which was also observed in the patient heterozygous for the C2693Y mutation, confirming that C2671 and C2693 are dispensable for dimerization (19). These two cysteines are most likely involved in intrachain bonds since they are located just outside the CK domain. To evaluate whether alterations of cysteines in this part of VWF have less impact on routing of VWF in general it would be interesting to compare the effects that loss of C2671 and C2693 have with the remaining five cysteines in this area.

The effect that the loss of an intrachain disulfide bond may have depends on its importance in the determination of the local and overall protein structure. If one assumes, *e.g.*, that the disulfide bond with C2671 only results in a minor change in the structure of VWF, then the effect on the level of VWF may be limited too. The compound heterozygous proband (C2671Y/deletion) with very low VWF:Ag (0.02-0.04 U/mL) showed increased levels of the VWF proteolysis products of 189, 176 and 140 kDa in patient plasma (50,51). These data suggested that other mechanisms than decreased synthesis and secretion of VWF, such as increased *in vivo* clearance and/or increased proteolysis of VWF may be involved in the development of the severe phenotype. The contribution of several aspects of clearance, such as ADAMTS13 cleavage, and survival of endogenous C2671Y VWF as well as recombinant C2671Y in a murine model was studied in **Chapter 3** of this thesis and will be discussed later.

The impact of amino acid identity on VWD phenotype

Strikingly, the majority of the cysteine mutations reported in the human VWF database (http://www.sheffield.ac.uk/vwf) are changed to bulky aromatic or large

charged amino acid residues (**Chapter 2b**). We hypothesized that the replacement of a cysteine residue with such amino acids may introduce disadvantageous effects on the conformation of the protein in addition to the loss of the cysteine bond. These changes may affect the biosynthesis and secretion of VWF.

To investigate this hypothesis we studied the differential effect of alteration of C2362 to alanine instead of phenylalanine (**Chapter 4**) (47) and of C2671 to alanine instead of tyrosine (**Chapter 2b**). We found that alteration of C2671 to an alanine indeed resulted in a slightly higher secretion of VWF compared to the C2671Y mutation (Chapter 2b). The observed decrease of intracellular retention of VWF was however not statistically significant. Both mutant proteins showed an indistinguishable multimer pattern on sodium dodecyl sulfate agarose gel electrophoresis. Apparently, loss of C2671 and its disulfide bond is the key contributor to the decreased level of VWF secreted and not necessarily the introduction of a tyrosine residue. Similar results were obtained for the C2362F and C2362A mutations (47), even though the C2362F mutation is not located in the same domain of VWF as C2671. The comparison of these two cysteines suggests that the introduced amino acid side chain does not introduce additional, detrimental conformational changes. However, the effect that alterations of cysteine residues to bulky, charged or small and uncharged residues have, may rely on the exact position of the cysteine in VWF and whether the cysteine in question forms an intrachain bond with a nearby or distant partner. A more general estimation of such effects would require the study of several cysteines at different positions and separate domains including alterations to an alanine, glycine and serine to allow a better understanding of a possible additional effect of introduced amino acid side chain.

Clearance of VWF

In **Chapter 3** we investigated whether missense mutations of cysteine residues could result in increased clearance. We showed that the discrepancy observed between the relatively high levels of recombinant C1130F, C1149R and C2671Y VWF secreted *in vitro* and the distinct decrease of plasma VWF:Ag observed *in vivo* (19) may be explained by increased clearance of mutant VWF (32). The increase in clearance was not explained by increased susceptibility to ADAMTS13 cleavage of either C1130F, C1149R or C2671Y VWF (Fig. 1) (32). Other mutations like the

R1205H and the S2179F mutation, which is located in the D4 domain of VWF, also result in increased clearance of VWF (Fig. 1) (52,53). This indicates that single point mutations in several specific regions of VWF may result in increased clearance of VWF possibly by allosteric effects on receptor-mediated clearance. Unfortunately, functional characterization of C1130F, C1149R, R1205H and C2671Y did not reveal any specific regions in VWF that could be involved in such enhanced clearance (32). However, increased clearance of VWF may still be an interesting contributor to the type 1 VWD phenotype as observed by Brown and co-workers (54). They assessed the half-life of VWF:Ag after infusion of DDAVP in patients with type 1 VWD or mild hemophilia A. Firstly, they found a two-fold reduction in the median half-life of VWF in the type 1 VWD patient group compared with the mild hemophilia A group. Secondly, they found lower baseline levels of VWF:Ag to be associated with a reduced half-life of VWF. This suggests that increased clearance may be the pathogenic mechanism in some cases of type 1 VWD. Further studies of those type 1 VWD patients and their families is necessary to reveal whether the increased clearance is due to a mutation in the VWF gene, the glycosylation status of VWF or whether it is a consequence of intrinsic high clearance of the patients. Infusion studies of VWF in these patients could exclude the latter as the causative factor. The contribution of alterations in the VWF gene can then be assessed in the murine model, possibly revealing specific regions to be involved in this mechanism. If no mutations are present, this indicates that, as observed for many type 1 VWD patients, other factors may be involved such as the glycosylation of VWF (ABO group, sialylation (55)) or other modifying loci. This may be the case in as many as one third of the patients, in which no VWF gene defect was detected in the MCMDM-1VWD study (28). There is an ongoing discussion whether these patients should be classified as type 1 VWD, since the low VWF levels are not correlated with mutations in the VWF coding sequence (28), but they are more likely the result of the large variation of VWF:Ag levels in the normal population (56) which is caused by ABO blood group and possibly some other loci.

Y1584C polymorphism in VWF

Alterations of amino acid residues that introduce or replace a cysteine residue result in an unpaired cysteine residue in VWF, since all cysteine residues in VWF

are normally involved in disulfide bonds. These unpaired cysteines may interfere with the normal paring scheme by the introduction of alternative disulfide bonds, thereby possibly affecting structure and function of the protein. In this view it is also interesting to evaluate the effects that introduction as well as loss of cysteines may have on expression, secretion and survival of VWF.

The alteration of tyrosine 1584 to a cysteine was initially detected in a type 2A VWD patient compound heterozygous for Y1584C and S1506L (57). Y1584C was reported to be a polymorphism rather than a candidate mutation, since it was found in three unaffected family members as well as in 3% of normal individuals (57) (Fig. 1). Recently, heterozygosity for this polymorphism has been found at a high frequency in affected participants in Canadian (14%), UK (25%) and European (8%) studies of type 1 VWD (28,29,58,59). However, the polymorphism has been detected at a low frequency in the general population in the Canadian (1%) and the European (0.4%) type 1 VWD studies (29,58). This difference in frequency suggests that Y1584C may be a candidate mutation rather than a polymorphism. The effect of this candidate mutation in relation to type 1 VWD is unclear; since co-expression experiments of the Y1584C mutation indicated only a mild effect on secretion and intracellular retention of VWF (58); since the Y1584C mutation does not always co-segregate with the type 1 VWD phenotype (58-60); and since increased susceptibility of VWF to proteolysis by ADAMTS13 co-segregates completely with the Y1584C mutation in unaffected as well as affected individuals (59,61). Even though this indicates that Y1584C may not be a causative mutation, it still may increase the risk of bleeding in type 1 VWD, especially in combination with blood group O which is prevalent among type 1 VWD patients (59,60).

To evaluate whether the Y1584C mutation itself is a mild risk factor for bleeding we screened cases and controls in the FACTors in ORal anticoagulant Safety (FACTORS) study for the presence of this mutation (**Chapter 6**) (62). The study includes patients on vitamin K antagonist (VKA) treatment that have (cases) and do not have (controls) experienced major bleeding and was initiated to search for novel risk factors associated with bleeding in VKA treated patients (63). However, due to the limited size of the study and the low frequency of the mutation we were not able to obtain a reliable estimate of the contribution of this mutation to the bleeding risk (62). Our results, however, do not exclude that this polymorphism contributes to the risk of bleeding in other patient categories.

It is intriguing that one individual with the Y1584C mutation and blood group O has the type 1 VWD phenotype whereas another does not (58). This suggests that other factors contribute to the difference in phenotype. Possibly the activity of the ADAMTS13 protease may differ between these individuals and tip the balance (64). It remains to be shown whether the Y1584C mutation and blood group O affect proteolysis of VWF in a negative manner under physiological circumstances. If so, it would be interesting to evaluate the contribution of these factors to the risk of bleeding and the protective effect they may have in development of arterial thrombosis and cardiovascular disease (64).

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Summary
&
Samenvatting
&
Sammanfattning

Summary

Von Willebrand factor (VWF) is a large plasma protein with essential functions in primary hemostasis. It is vital for the generation of a platelet plug as it has the ability to adhere to exposed extracellular matrix such as collagens at the site of injury and to then capture platelets. This leads to adhesion and subsequent aggregation of the platelets generating a seal of the injured area. This plug is reinforced by the insoluble fibrin fibres which are generated via localized activation of the coagulation cascade. Although VWF itself is not directly involved in this cascade it is important for this process as it functions as a carrier of factor VIII which is an important co-factor in this cascade. Loss of functional VWF leads to a bleeding disorder called von Willebrand disease (VWD). The severity of this disease varies widely and depends on the type of VWF defect. VWD is divided into quantitative and qualitative VWF defects. Type 1 VWD corresponds to partial loss of VWF, while type 3 VWD corresponds to almost complete loss of VWF. Type 2 VWD encompasses all qualitative dysfunctions of VWF.

VWF contains a large number of cysteine residues which all form either intrachain or interchain disulfide bonds. Intrachain disulfide bonds are important for the structure of the individual subunit, the monomer, while interchain disulfide bonds are crucial for the generation of high molecular weight (HMW) multimers. In the endoplasmic reticulum (ER), two proVWF monomers are linked *via* interchain disulfide bonds between the cystine knot (CK) domains (the 90 C-terminal amino acids) to form dimers. HMW multimers of VWF are formed in the Golgi where proVWF dimers are linked *via* interchain bonds between cysteine residues in the D3 domain of VWF. The multivalency of this protein is of utmost importance for its function as a molecular glue between exposed subendothelial structures and the rapidly passing platelets. Ultra-large VWF multimers are stored in α-granules of platelets or in Weibel-Palade bodies of endothelial cells and can be secreted on demand. A detailed introduction to the biosynthesis of VWF, its structure and function, its role in hemostasis and a description of the different types of VWD is given in **Chapter 1**.

The main goal of the research described in this thesis was to investigate the effect that missense mutations of cysteine residues in different domains of VWF have on dimerization and multimerization of VWF, how these mutations

influence the intracellular routing and secretion of VWF, and how this can result in the generation of quantitative or qualitative VWF defects.

In **Chapters 2-5** the effects of mutated cysteines in different domains of VWF were studied by expression of the full-length human VWF protein in a human kidney cell line, 293T cells. In all of these studies single transfections as well as co-transfections of the wild-type (wt) and mutant constructs were performed. Subsequently, the quantity and quality of the secreted and the intracellular recombinant VWF protein were analyzed by ELISA and SDS-agarose electrophoresis and in some instances VWF was reduced and separated by SDS-polyacrylamide gel electrophoresis.

In **Chapter 2a** the effects of five cysteine mutations, that all were found in patients with a quantitative defect of VWF, were investigated. The effects of these mutations on dimerization and multimerization and the quantity of produced VWF are described in relation to the characteristics of the patients. The results obtained in single transfection and co-transfections of wt and either C2739Y or C2754W agreed with the VWD phenotype observed in patients with these mutations. This shows that missense mutations of cysteine residues in the CK domain of VWF can result in a quantitative VWF defect, and mimic the phenotype of a null allele. This was, however, not the case for the C1130F, C1149R and C2671Y mutations. In our cell system these mutations had a milder effect on secretion which did not agree with the low levels of VWF antigen (VWF:Ag) detected in patients with these mutations.

Having shown that the low levels of VWF:Ag observed in patients with C1130F, C1149R or the C2671Y mutation was not due to decreased synthesis, and could not be explained completely by the decrease in secreted VWF or intracellular retention of VWF, we explored the possibility of increased clearance of the mutant VWF protein. In **Chapter 3** we investigated the sensitivity of recombinant VWF to the VWF-cleaving protease (ADAMTS13) in an *in vitro* system using urea as an unfolding agent of VWF. In addition, we estimated the *in vivo* survival of the mutant proteins in patients after infusion of 1-deamino-8-D-arginine vasopressin (DDAVP) and of recombinant mutant VWF in a VWF-deficient mouse model. Several parameters pointed to an increase in the clearance of the mutant proteins both in the patients and the mouse model. The increased ratio between VWF-propeptide and mature VWF, its reduced survival, and the four-fold increased clearance rate of the mutants in mice appeared not to

be linked with an increase in sensitivity to proteolysis by ADAMTS13. We were unable to identify any common functional or structural characteristics between the different mutant proteins that could be associated with increased clearance of VWF.

In several Italian families with autosomal recessive, severe VWD with a predominantly quantitative VWF defect the C2362F mutation has been identified (**Chapter 4**). The low level of secreted mutant protein and the strong intracellular retention of C2362F VWF *in vitro* could readily explain the quantitative VWF defect observed in patients carrying this mutation. The two-fold decrease in half-life of C2362F VWF observed *in vivo* was, however, not explained by increased sensitivity of the mutant protein to ADAMTS13 *in vitro*. The contribution of clearance mechanisms, indicated by the reduced half-life of C2362F, to the VWD phenotype appeared minimal and the VWF defect is most likely the result of impaired secretion and intracellular retention and rapid degradation of the misfolded mutant protein.

We noted that most cysteine mutations identified in VWD patients are alterations to bulky amino acids with a charged or a hydrophobic side chain. To investigate whether such amino acid side chains induce effects on the conformation of the VWF subunit in addition to those generated by the loss of the disulfide bond *per se*, the alanine variants of the C2671Y (**Chapter 2b**) and the C2362F (**Chapter 4**) VWF mutants were expressed. Only minor differences in secretion and intracellular levels of C2671Y and C2362F *vs* respectively C2671A and C2362A were observed. Thus for cysteines at these positions in VWF the quantitative VWF defect observed in the patients could be attributed to the loss of their respective disulfide bonds.

We also noted that alterations of cysteines in the CK domain of VWF, which result in a dimerization defect of VWF, have been identified in both qualitative type 2A (IID) and quantitative type 3 VWD (**Chapter 2a**). We hypothesized that this difference in phenotype is related to whether the mutated cysteine residue is involved in the formation of an interchain or intrachain disulfide bond (**Chapter 5**). The effect of the novel 2A(IID) VWD mutation, C2773S, on quantity and quality of VWF was compared with the effects of two type 3 VWD mutations, C2739Y and C2754W. In contrast to the C2739Y and C2754W mutations, the C2773S mutation did not affect the quantity of secreted VWF. This showed that loss of either one of the intrachain bonds formed by cysteines 2739 and 2754 have a more

profound effect on the conformation of the VWF subunit than loss of the interchain disulfide bond formed by cysteine 2773. C2773S VWF did, however, have a major effect on the multimerization process of wt VWF due to N-terminal multimerization of C2773S proVWF monomers to wt proVWF dimers. This hindered further multimerization of wt proVWF dimers and the generation of HMW multimers. This ultimately results in the typical 2A(IID) VWD multimer pattern with the occurrence of odd-numbered multimers in between even-numbered multimers, and the lack of HMW multimers that was observed in co-transfections of C2773S and wt VWF constructs. This suggests that loss of a single disulfide bond in the CK domain of VWF will lead to a recessive, quantitative VWF deficiency if an intrachain disulfide bond is involved, and to a dominant negative, qualitative defect of VWF if an interchain disulfide bond is involved.

In **Chapter 6**, we screened cases and controls in the FACTORS (FACTors in ORal anticoagulation Safety) for the presence of the Y1584C VWF polymorphism. This study was initiated to search for novel risk factors that contribute to the risk of bleeding in patients that are treated with vitamin K antagonists. The Y1584C VWF polymorphism has been reported at high frequency in patients with type 1 VWD and is associated with rapid proteolysis by ADAMTS13 in an *in vitro* assay. We found a first indication that the Y1584C VWF polymorphism may be associated with bleeding in patients treated with vitamin K antagonists. Unfortunately, the limited size of the study and the low frequency of Y1584C did not allow a reliable estimate of the contribution of this polymorphism to the risk of bleeding in this patient group.

Finally, in **Chapter 7** the results obtained in our studies are discussed in a broader perspective and placed against a background of results reported by others. Our studies of the effects of mutated cysteine residues in VWF have contributed to the steadily increasing knowledge of the effects that mutated cysteine residues in VWF may have on expression, secretion and intracellular retention, processing, and clearance of VWF in relation to the phenotype described in the patients carrying these mutations.

Samenvatting

De von Willebrand factor (VWF) is een groot eiwit met belangrijke functies in de primaire hemostase. VWF is essentieel voor de vorming van een bloedplaatjes plug doordat het op de plaats van de verwonding zowel aan de vrijgekomen extracellulaire matrix, zoals collageen, als aan bloedplaatjes kan binden. Dit leidt tot adhesie en vervolgens aggregatie van de bloedplaatjes waardoor uiteindelijk de verwonding afgedekt wordt. De plaatjes plug wordt vervolgens versterkt door fibrinedraden die door gelocaliseerde activatie van de stollingscascade ontstaan. VWF is zelf niet direct betrokken bij deze cascade maar levert wel een belangrijke bijdrage als drager van factor VIII, dat wel een belangrijke functie als co-factor in deze cascade vervult. Gebrek aan functioneel VWF kan leiden tot een bloedingsziekte, genaamd de ziekte van von Willebrand. De ernst van deze ziekte varieert sterk en is afhankelijk van het type van het VWF defect. De ziekte van von Willebrand wordt onderverdeeld in kwantitative en kwalitatieve VWF defecten. Bij type 1 ziekte van von Willebrand is VWF gedeeltelijk afwezig, terwijl type 3 correspondeert met een bijna volledige afwezigheid van het eiwit. Type 2 ziekte van von Willebrand omvat alle kwalitative defecten van de VWF.

VWF bevat een groot aantal cysteïneresiduen die allemaal zwavelbruggen vormen. De zwavelbruggen zijn onderverdeeld in bruggen binnen de VWF subeenheid (*intra*moleculaire zwavelbruggen) en bruggen tussen verschillende subeenheden van VWF (*inter*moleculaire zwavelbruggen). De intramoleculaire zwavelbruggen zijn van groot belang voor het behoud van de structuur van de individuele subeenheid van VWF, de monomeer. De intermoleculaire zwavelbruggen zijn noodzakelijk voor het genereren van multimeren van hoogmoleculair gewicht (HMG). De 90 C-terminale aminozuren van VWF vormen het zogenaamde Cysteïne Knoop (CK) domein. In het endoplasmatisch reticulum (ER) worden proVWF dimeren gevormd door intermoleculaire zwavelbruggen tussen de CK domeinen van twee proVWF moleculen. In het Golgi worden HMG multimeren van VWF gevormd door het verbinden van proVWF dimeren *via* intermoleculaire zwavelbruggen tussen cysteïneresiduen in het D3 domein van VWF. Het repetitieve karakter van VWF is zeer belangrijk voor de functie als moleculaire lijm tussen vrijgekomen subendotheliale structuren en de snel passerende bloedplaatjes. VWF multimeren van zeer hoog molecuulgewicht worden in de α-granulae van bloedplaatjes of in Weibel-Palade lichaampjes van

endotheliale cellen opgeslagen en worden naar behoefte uitgescheiden. In **Hoofdstuk 1** wordt de biosynthese van VWF, de structuur en functie, de rol in de primaire hemostase en de verschillende types van de ziekte van von Willebrand in detail beschreven.

Het hoofddoel van het onderzoek beschreven in dit proefschrift was het bestuderen van de effecten die missensemutaties van cysteïneresiduen in verschillende domeinen van VWF hebben op dimerisatie en multimerisatie van VWF, hoe deze mutaties de intracellulaire routing en secretie van VWF beïnvloeden, en hoe deze mutaties tot kwantitative en kwalitative WVF defecten kunnen leiden.

In **Hoofdstukken 2-5** werden de effecten van veranderde cysteïnes in verschillende domeinen van VWF bestudeerd door humaan VWF in menselijke 293T niercellen tot expressie te brengen. In alle studies zijn zowel transfecties met enkele constructen als co-transfecties van wildtype (wt) en mutante constructen uitgevoerd. De kwantiteit en kwaliteit van het uitgescheiden en intracellulaire recombinant VWF werden vervolgens bestudeerd door middel van ELISA en SDS-agarose elektroforese, en in een aantal gevallen werd VWF gereduceerd en gescheiden door SDS-polyacrylamide gel elektroforese.

In **Hoofdstuk 2a** werden de effecten onderzocht van vijf cysteïnemutaties, die allemaal in patiënten met een kwantitatief VWF defect zijn gevonden. De effecten van deze mutaties op dimerisatie en multimerisatie, en de kwantiteit van geproduceerd VWF werden beschreven in relatie tot de eigenschappen van de patiënten met die mutaties. De resultaten van transfecties met een enkel construct en co-transfecties van wt met C2739Y of C2754W kwamen overeen met het fenotype van patiënten met deze mutaties. Dit laat zien dat missense mutaties van cysteïneresiduen in het CK domein van VWF een kwantitatief defect kunnen veroorzaken en het fenotype van een nul allel kunnen nabootsen. Dit was niet het geval voor de C1130F, C1149R en C2671Y mutaties. In ons celsysteem hadden deze mutaties een milder effect op de secretie van VWF, hetgeen niet geheel overeenkwam met het lage niveau van VWF antigeen (VWF:Ag) in patiënten met dezelfde mutaties.

Nadat we hadden laten zien dat het lage niveau van VWF:Ag in de patiënten met de C1130F, C1149R of de C2671Y mutatie niet veroorzaakt werd door verlaagde synthese en niet geheel uitgelegd kon worden door de verlaagde secretie van VWF of intracellulaire retentie van VWF, onderzochten wij de

mogelijkheid van versnelde klaring van de mutante VWF eiwitten. In **Hoofdstuk 3** werd de gevoeligheid van mutant VWF voor het VWF-splitsende protease (ADAMTS13) onderzocht in een *in vitro* test met ureum als ontvouwings reagens van VWF. Verder werd de halfwaardetijd van mutante VWF eiwitten *in vivo* bepaald door middel van intraveneuze toediening van 1-deamino-8-D-arginine vasopressin (DDAVP) in patiënten met de mutaties en uit de overleving van mutant recombinante VWF in VWF-deficiënte muizen. Meerdere parameters wezen op een verhoogde klaring van het mutante VWF zowel in de patiënten alsook in het muismodel. De verhoogde verhouding tussen VWF-propeptide en matuur VWF, de verkorte overleving van mutant VWF, en de viermaal hogere klaringssnelheid van de mutanten in de muizen bleek niet gerelateerd te zijn aan een verhoogde gevoeligheid voor proteolyse door ADAMTS13. Wij konden geen gemeenschappelijke functionele of structurele karakteristieken tussen de verschillende mutanten aantonen die met verhoogde klaring van VWF geassocieerd zouden kunnen zijn.

In meerdere Italiaanse families met een ernstige vorm van de ziekte van von Willebrand, die autosomaal recessief overgeërfd wordt en voornamelijk in een kwantitatief VWF defect resulteert, is de C2362F mutatie gevonden (**Hoofdstuk 4**). Het lage niveau van uitgescheiden mutant eiwit en de hoge intracellulaire retentie van C2362F VWF *in vitro* kan het kwantitatief VWF defect, dat in patiënten met deze mutatie wordt gevonden, helemaal verklaren. De tweevoudig verlaagde *in vivo* halfwaardetijd van C2362F VWF lijkt niet veroorzaakt te zijn door verhoogde gevoeligheid van C2362F VWF voor ADAMTS13 *in vitro* en suggereert dat gestoorde klaring een bijdrage kan leveren aan het fenotype van de ziekte van von Willebrand. Die bijdrage leek echter beperkt en het lage VWF gehalte is waarschijnlijk het resultaat van verminderde secretie in combinatie met intracellulaire retentie en versnelde afbraak van het verkeerd gevouwen eiwit.

Het viel ons op dat de meeste cysteïnemutaties die in patiënten zijn gevonden veranderingen naar grote aminozuren met geladen of met hydrofobe zijketens zijn. Om te onderzoeken of zulke aminozuurzijketens een extra invloed hebben op de conformatie van de VWF subeenheid naast de effecten, die het verlies van de zwavelbrug op zich hebben, werden de alaninevarianten van de C2671Y (**Hoofdstuk 2b**) en de C2362F (**Hoofdstuk 4**) VWF mutanten tot expressie gebracht. Er werden alleen kleine verschillen in secretie en intracellulaire niveaus van C2671Y en C2362F VWF geobserveerd vergeleken met de alaninevarianten.

Dus voor cysteïne mutaties in deze posities in VWF kan het kwantitatieve VWF defect worden uitgelegd door de afwezigheid van de respectievelijke zwavelbruggen.

Wij merkten ook op dat gerapporteerde veranderingen van cysteïnes in het CK domein van VWF die tot dimerisatie defecten van VWF leiden geïdentificeerd zijn in zowel kwalitatief type 2A(IID) ziekte van von Willebrand als kwantitatief type 3 ziekte van von Willebrand (**Hoofdstuk 2a**). Wij postuleerden dat dit verschil in het fenotype van de ziekte van von Willebrand veroorzaakt wordt door de betrokkenheid van het gemuteerde cysteïneresidu bij een inter- of intramoleculaire zwavelbrug in VWF (**Hoofdstuk 5**). Het effect van de door ons gevonden type 2A(IID) ziekte van von Willebrand mutatie, C2773S, op kwantiteit en kwaliteit van VWF werd vergeleken met de effecten van twee type 3 ziekte van von Willebrand mutaties, C2739Y en C2754W. In tegenstelling tot de C2739Y en C2754W mutaties, resulteerde de C2773S mutatie niet in een kwantitatief effect op VWF secretie. Dit wijst erop dat verlies van elke van de intramoleculaire zwavelbruggen die cysteïnes 2739 en 2754 vormen een sterker effect heeft op de conformatie van de VWF subeenheid dan het verlies van de intermoleculaire zwavelbrug gevormd door cysteïne 2773. De C2773S VWF mutatie had echter wel een groot effect op de multimerisatie van wt VWF door de N-terminale multimerisatie van C2773S proVWF monomeren aan wt proVWF dimeren. Dit verhinderde verdere multimerisatie van wt proVWF dimeren en de productie van HMG multimeren. Dit resulteert uiteindelijk in het kenmerkende 2A(IID)-multimerenpatroon dat bestaat uit multimeren opgebouwd uit een oneven aantal monomeren naast multimeren met een even aantal monomeren, en uit de afwezigheid van HMG multimeren zoals dat werd waargenomen in co-transfecties van C2773S en wt VWF constructen. Dit suggereert dat verlies van één zwavelbrug in het CK domein van VWF resulteert in een recessief, kwantitatief VWF defect wanneer het een intramoleculaire zwavelbrug betreft, en in een dominant-negatief, kwalitatief defect van VWF wanneer het een intermoleculaire zwavelbrug betreft.

In **Hoofdstuk 6**, zochten wij in het patiënt-controle onderzoek FACTORS (FACToren in de veiligheid van ORale antistollingsmiddelen) naar de aanwezigheid van het Y1584C-VWF-polymorfisme. Deze studie was opgezet om nieuwe risicofactoren te identificeren die bijdragen aan het risico op bloedingen in patiënten, die worden behandeld met vitamine-K-antagonisten. Het Y1584C-

VWF-polymorfisme blijkt vaak voor te komen in patiënten met type 1 ziekte van von Willebrand en gaat gepaard met versnelde proteolyse van VWF door ADAMTS13 in een *in vitro* test. Wij vonden een milde aanwijzing dat bloedingen in patiënten, die behandeld worden met vitamine-K-antagonisten, geassocieerd zijn met het Y1584C-polymorfisme. Helaas maakte de beperkte omvang van de studie en de lage frequentie van Y1584C het niet mogelijk om een betrouwbare schatting te maken van de bijdrage van dit polymorfisme aan het risico op bloedingen in deze patiënten groep.

Ten slotte werden in **Hoofdstuk 7** onze resultaten in een breder perspectief geplaatst en gespiegeld aan de bevindingen van andere onderzoeken. Onze studies van de effecten van gemuteerde cysteïneresiduen in VWF hebben bijgedragen aan de gestaag toenemende kennis van de effecten die veranderingen van cysteïneresiduen in VWF hebben op expressie, secretie en intracellulaire retentie, processing, en klaring van VWF in relatie tot de fenotypes beschreven in patiënten met dezelfde mutaties.

Sammanfattning

Von Willebrand faktor (VWF) är ett stort protein med viktiga funktioner i primära hemostasen. VWF är väsentlig för bildandet av en blodplättsplugg genom att binda molekyler som frilagts i den skadade blodkärlsbädden, och genom att binda blodplättar. Detta leder till adhesion av, och följaktligen aggregation av blodplättarna vilket resulterar i att det skadade området täcks. Blodplättspluggen förstärks av fibrintrådar som bildas genom lokal aktivering av koagulations-kaskaden. VWF deltar inte direkt i denna kaskad men är viktigt genom sin funktion som bärare av faktor VIII som har en viktig funktion som kofaktor i denna kaskad. Brist på fungerande VWF kan orsaka blödarsjukan von Willebrands sjukdom. Sjukdomens allvarlighetsgrad varierar och beror på vilken typ av VWF-defekt patienten har. Von Willebrands sjukdom indelas i kvantitativa och kvalitativa VWF-defekter. Typ 1 von Willebrands sjukdom karakteriseras av partiell brist på VWF, medan typ 3 von Willebrands sjukdom innebär nästintill fullständig avsaknad av proteinet. Typ 2 von Willebrands sjukdom omfattar alla sorters kvalitativa defekter av VWF.

VWF innehåller ett stort antal av aminosyran cystein vilka alla bildar kovalenta svavelbryggor. Svavelbryggorna är fördelade i bindningar inom VWF-molekylen, så kallade *intra*molekylära svavelbryggor, och bindningar mellan enskilda VWF-molekyler, så kallade *inter*molekylära svavelbryggor. De intramolekylära svavelbryggorna är mycket viktiga för formen hos de enskilda VWF-molekylerna, så kallade monomerer. De intermolekylära svavelbryggorna är oumbärliga för bildandet av VWF-multimerer med hög molekylär vikt (HMV). De 90 mest C-terminala aminosyrorna av VWF bildar den så kallade cysteinknutdomänen (CK-domänen) i VWF. I det endoplasmatiska retikelet (ER) bildas proVWF-dimerer *via* intermolekylära svavelbryggor mellan cysteiner belägna i CK-domänerna i två proVWF-molekyler. I Golgi-apparaten bildas VWF-multimerer med HMV *via* intermolekylära svavelbryggor mellan cysteiner i D3-domänen i proVWF-dimerer. Den repetetiva karaktären hos VWF med HMV är mycket viktigt för proteinets funktion som molekylärt klister mellan frilagda subendoteliala strukturer och de snabbt förbipasserande blodplättarna. VWF-multimerer med särdeles hög molekylärvikt lagras i α-granula i blodplättar eller i Weibel-Palade-kroppar i endotelceller och utsöndras vid behov. I **Kapitel 1**

beskrivs biosyntesen av VWF, dess struktur och funktion, dess roll i den primära hemostasen och de olika typerna av von Willebrands sjukdom i detalj.

Syftet med studierna som beskrivs i denna avhandling var att undersöka effekterna som förändringar av cysteiner belägna i olika delar av VWF har på proteinets förmåga att bilda dimerer (dimerisera) och att bilda multimerer (multimerisera), samt att se hur dessa förändringar (mutationer) påverkar syntesen, proteinets intracellulära routing och utsöndringen av VWF, och hur dessa mutationer kan orsaka kvantitativa och kvalitativa VWF-defekter.

I **Kapitel 2-5** beskrivs effekterna av förändrade cysteiner i olika domäner av VWF genom att uttrycka humant VWF i 293T celler som är njurceller av mänskligt ursprung. I alla studier utfördes både transfektioner av 293T celler med ett konstrukt (enkla transfektioner) och med en kombination av ett konstrukt med normal VWF med ett konstrukt med mutant VWF (co-transfektioner). Kvantiteten och kvaliteten av utsöndrad och intracellulär rekombinant VWF studerades med hjälp av ELISA och SDS-agarose gelelektrofores och i vissa fall reducerades VWF och skildes genom SDS-polyakrylamid gelelektrofores.

I **Kapitel 2a** redogörs för effekterna utav fem cysteinmutationer, vilka alla har identifierats i patienter med en kvantitativ VWF-defekt. Effekterna av dessa mutationer på dimerisering och multimerisering, och kvantiteten av det producerade VWF jämfördes med de egenskaper VWF har i patienter med dessa mutationer. Resultaten från transfektioner med enkla konstrukt och sådana från co-transfektioner av normal VWF i kombination med C2739Y eller C2754W stämde bra överens med fenotypen hos patienter med dessa mutationer. Detta visar att förändringar av cysteiner till en annan aminosyra (missense mutation) i CK-domänen av VWF kan förorsaka en kvantitativ VWF defekt som liknar effekten av en noll allel (en mutation som förhindrar produktion av protein). Detta var inte fallet för mutationerna C1130F, C1149R och C2671Y. I vårt cellsystem hade dessa mutationer en mildare effekt på utsöndringen av VWF, vilket inte stämde överens med den låga nivån av VWF-antigen (VWF:Ag) i plasma hos patienter med samma mutationer.

Efter det att vi hade visat att den låga nivån av VWF:Ag i patienter med förändringar av C1130, C1149 eller C2671 inte orsakas av minskad syntes av VWF, och inte kunde förklaras av den något lägre utsöndringen av VWF eller intracellulär retention av VWF undersökte vi ifall den låga VWF:Ag nivån i patienterna orsakades av ökad omsättning av de förändrade proteinerna. I

Kapitel 3 beskrivs hur känsligheten av förändrat VWF för det VWF-klyvande proteaset (ADAMTS13) undersöktes i ett test *in vitro* med ureum som denatureringsreagens. Dessutom bestämdes halveringstiden för de förändrade VWF-proteinerna (C1130F, C1149R och C2671Y) *in vivo* genom intravenös infusion av 1-deamino-8-D-arginine vasopressin (DDAVP) i patienter med dessa mutationer, och genom injektion av mutant rekombinant VWF i möss som ej syntetiserar egen VWF. Flera parametrar pekade mot en ökad omsättning av mutant VWF både i patienterna och i musmodellen. Det förhöjda förhållandet mellan VWF-propeptid och VWF, den förkortade halveringstiden av mutant VWF, och den fyra gånger högre omsättningen av mutant protein i mössen var inte kopplade till en ökad känslighet för proteolys av ADAMTS13 *in vitro*. Vi kunde inte upptäcka en gemensam funktionell eller strukturell faktor mellan de olika mutanterna som var direkt kopplad till den ökade omsättningen av VWF.

I flera Italienska familjer med en allvarlig form av von Willebrands sjukdom, som ärvs autosomalt och recessivt och huvudsakligen resulterar i en kvantitativ VWF-defekt, har mutationen C2362F identifierats (**Kapitel 4**). Den låga nivån av utsöndrad C2362F VWF och den höga intracellulära nivån av C2362F VWF *in vitro* kan helt förklara den kvantitativa VWF-defekten som observerats i patienter med denna mutation. Den förkortade halveringstiden av C2362F VWF *in vivo* verkar inte vara resultatet av en förhöjd känslighet av C2362F VWF för ADAMTS13 vilket indikerar att en störning av omsättningen av VWF bidrar till fenotypen. Detta bidrag verkar dock vara begränsat och den låga VWF-nivån är troligtvis resultatet av en minskad utsöndring, intracellulär retention och förmodligen snabb nedbrytning av C2362F VWF på grund av dess felaktiga konformation.

Vi lade märke till att de flesta förändringar av cysteiner i VWF som identifierats i patienter med von Willebrands sjukdom är förändringar till aminosyror med stora laddade eller hydrofoba sidokedjor. För att undersöka om dessa sidokedjor har en extra effekt på konformationen av VWF-monomeren, utöver den effekt som förlusten av svavelbryggan har, studerades alaninvarianterna av C2671Y (**Kapitel 2b**) och av C2362F (**Kapitel 4**). Endast små skillnader i utsöndring och intracellulära nivåer av C2671Y och C2362F VWF observerades i jämförelse med deras respektive alaninvarianter. För cysteiner i dessa positioner i VWF kan den kvantitativa VWF-defekten förklaras av frånvaron av deras respektive svavelbryggor.

Vi lade även märke till att de funna förändringarna av cysteiner i CK-domänen av VWF som leder till dimeriseringsdefekter i VWF kan orsaka både kvalitativ typ 2A(IID) von Willebrands sjukdom och kvantitativ typ 3 von Willebrand sjukdom (**Kapitel 2a**). Vi postulerade att denna skillnad i sjukdomsfenotyp beror på om det muterade cysteinet deltar i en inter- eller intramolekylär svavelbrygga i VWF (**Kapitel 5**). Effekten av den av oss funna typ 2A(IID) mutationen, C2773S, på kvantitet och kvalitet av VWF jämfördes med effekterna av två typ 3 von Willebrand sjukdoms mutationer, C2739Y och C2754W. I motsats till resultaten för mutationerna C2739Y och C2754W resulterade C2773S mutationen inte i en kvantitativ effekt på utsöndringen av VWF. Detta visar att förlust av en av de intramolekylära svavelbryggor som cystein 2739 eller 2754 ingår i har en starkare effekt på konformationen av VWF-monomeren än förlusten av den intermolekylära svavelbryggan som cystein 2773 deltar i. VWF-mutationen C2773S har dock en stark effekt på normal VWF:s förmåga att bilda multimerer genom N-terminal multimerisering utav C2773S VWF-monomerer till normala proVWF-dimerer. Detta förhindrar vidare multimerisering av normal proVWF-dimerer och produktionen av HMV-multimerer. Slutligen resulterar detta i det multimermönster som är karakteristiskt för 2A(IID) von Willebrands sjukdom. Detta mönster består av multimerer med ett ojämnt antal monomerer, förutom multimerer bestående av ett jämnt antal monomerer, och karakteriseras av brist på HMV-multimerer. Det typiska 2A(IID) mönstret observerades i co-transfektioner av normal och C2773S VWF och visar att förlust av en svavelbrygga i CK-domänen utav VWF resulterar i en recessiv, kvantitativ VWF-defekt vid förlust av en intramolekylär svavelbrygga medan det resulterar i en dominant-negativ, kvalitativ defekt vid förlust av en intermolekylär svavelbrygga.

I **Kapitel 6**, rapporteras resultaten från vår undersöking av patient-kontroll studien FACTORS (FAKTorer i säkerhet av ORal antikoagulationsmedel) för närvaro av Y1584C VWF-polymorfismen. Denna studie initierades för att identifiera nya riskfaktorer som bidrar till risken för blödning i patienter som behandlas med vitamin K-antagonister. Y1584C VWF-polymorfismen förekommer frekvent hos patienter med typ 1 von Willebrands sjukdom och leder till en snabbare proteolys av VWF genom ADAMTS13 *in vitro*. Vi fann endast en svag indikation att blödningar i patienter som behandlas med vitamin K-antagonister är associerad med denna polymorfism. Tyvärr gjorde den ringa storleken på

studien och den låga frekvensen av Y1584C att det var omöjligt att trovärdigt uppskatta bidraget av denna polymorfism till risken för blödning i denna patientgrupp.

Slutligen diskuteras våra resultat i ett bredare perspektiv i **Kapitel 7** där de speglas gentemot andra forskares resultat. Våra studier har bidragit till en ökad kunskap om de effekter som förändringar av aminosyran cystein i VWF har på syntesen, utsöndring och intracellulär retention, processing, och omsättning av VWF i förhållande till fenotypen beskriven hos patienter med samma mutationer.

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Curriculum Vitae

Anna Pernilla Tjernberg was born on the 20th of December 1969 on the Isle of Alnö (Sweden). In 1988, she obtained the upper level school β certificate at Katrinelunds Gymnasium (Sundsvall, Sweden). She studied at the Sundsvall Vårdhögskola (Sundsvall, Sweden) for two and half years and obtained a diploma as a technician in clinical chemistry. During 1993-1994 she studied at Mitthögskolan (Midsweden University, Sundsvall, Sweden) and continued her studies at the Uppsala University (Uppsala, Sweden).

At the end of her study in Uppsala she participated in an Erasmus exchange program with Leiden University (Leiden, the Netherlands). For seven months she did her major at the Plantvirology laboratory of Prof. dr. J.F. Bol under supervision of Dr. G.E.M. Abbink. She investigated the involvement of the helicase of tobacco mosaic virus in hypersensitive response in plants. Autumn 1998 she returned to Uppsala to successfully defend her thesis and obtained the Master of Science degree in Chemistry at Uppsala University in march 1999.

Spring 1999 she moved to Leiden, the Netherlands and mid June she started as a Ph.D. student in the group of Prof. dr. R.M. Bertina at the Leiden University Medical Center, Leiden (LUMC). Under supervision of Dr. H.C.J. Eikenboom she has investigated the role of cysteine residues in von Willebrand factor. The results are described in this thesis.

Until April 2006 the author has been working as a Scientist at Chromagenics/Crucell to improve stable expression of therapeutic antibodies in mammalian cell lines.